IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Laurence J. Zwiebel
Serial No.:	10/056,405
Filed:	March 8, 2002
For:	Arrestin Gene, Polypeptide, and Methods of Use Thereof
Group No.	1647
Examiner:	Jon M. Lockard
Attorney's Docket No.	N7841

DECLARATION OF LAURENCE J. ZWIEBEL UNDER 37 C.F.R. §1.132

I, Laurence J. Zwiebel, declare and state as follows:

1. I am the applicant in the above-referenced patent application.

2. I am currently a Full Professor at Vanderbilt University in the Department of Biological Sciences. I joined Vanderbilt University as an Assistant Professor in 1998. For the past 12 years, I have studied insect olfaction cascades. During my research career, I have studied insect related molecular biology since 1986. I received a Doctorate of Philosophy in Molecular Biology from Brandeis University, Waltham, Massachusetts, in 1992.

3. I am familiar with SEQ ID NO: 2, *Anopheles gambiae* arrestin 1 (AgArr1), and the experimental protocols set forth herein. Under my direction and control, the results discussed herein were obtained by use of laboratory protocols as set forth hereinbelow. The results shown in Figures 1-4 summarize the ability of SEQ ID NO: 2, *Anopheles gambiae* arrestin 1 (AgArr1), to functionally rescue olfactory defects that have previously been shown specifically due to deficits in the *Drosophila melanogaster* arrestin 1 gene (DmArr1). Stated another way, these experiments constitute a replacement of DmArr1 with AgArr1 in *Drosophila*

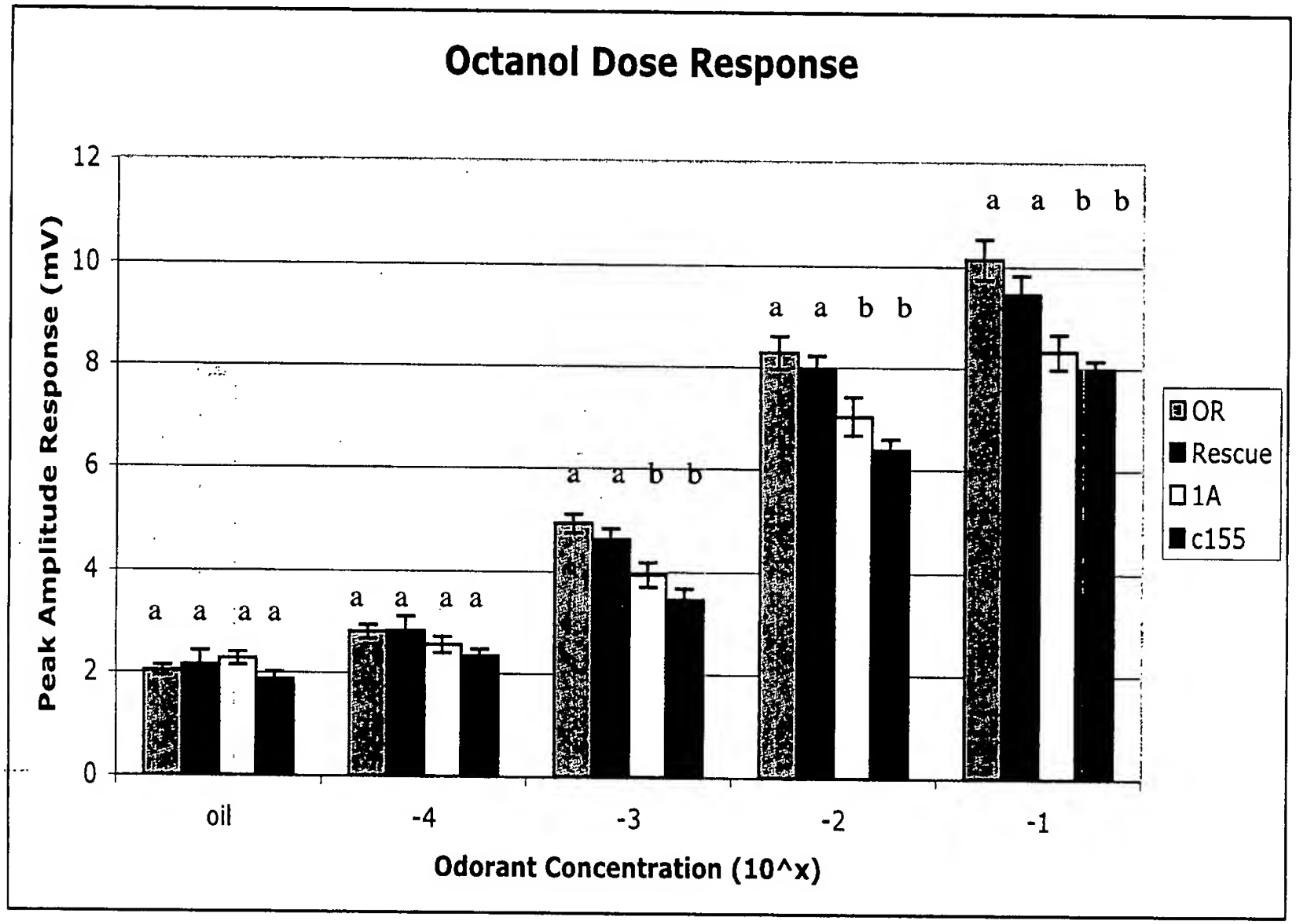
olfactory physiology and thereby, in my opinion, constitute *pro-forma* proof of the ability of AgArr1 to function as an olfactory arrestin.

4. These experiments use transgenic approaches to direct the expression of AgArr1 to *Drosophila* olfactory neurons as well as the electroantennogram technique (Alcorta, E., 1991, Characterization of the electroantennogram in *Drosophila melanogaster* and its use for identifying olfactory capture and transduction mutants. *Journal of Neurophysiology 65*, 702-714) to provide an electrophysiological assessment of olfactory responses to four different odorant stimuli (octanol, butanol, heptanoic acid and octyl acetate, shown in Figures 1-4, respectively) across the antennae of *Drosophila melanogaster*. As shown in Figures 1-4, "Rescue" flies (i.e. those expressing AgArr1) return to wild-type (wild-type flies marked as "OR") levels of olfactory responses for several odorants whilst mutant parental lines ("c155" flies and "1A" flies) consistently maintain olfactory responses that are characteristic of flies carrying DmArr1 arrestin mutations. These experiments were conducted as described in Merrill, C. E., Sherertz, T. M., Walker, W. B., and Zwiebel, L. J. (2005). Odorant-specific requirements for arrestin function in Drosophila olfaction. *J Neurobiol 63*, 15-28, a copy of which is attached hereto.

5. These studies replicate similar experiments that originally characterized the olfactory deficits associated with DmArr1 mutations (Merrill, C. E., Riesgo-Escovar, J., Pitts, R. J., Kafatos, F. C., Carlson, J. R., and Zwiebel, L. J. (2002). Visual arrestins in olfactory pathways of Drosophila and the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci* U S A *99*, 1633-1638) as well as more recent studies that were carried out using DmArr1 to rescue its own mutant phenotype (Merrill, C. E., Sherertz, T. M., Walker, W. B., and Zwiebel, L. J. (2005). Odorant-specific requirements for arrestin function in Drosophila olfaction. *J Neurobiol 63*, 15-28). In my opinion, these latter studies constitute a *pro-forma* demonstration that olfactory deficits were due to defects in DmArr1 expression and function.

FIGURE 1

Octanol



Letters indicate statistically distinct

or

similar response groups

OR, n=11

Resuce, n=15

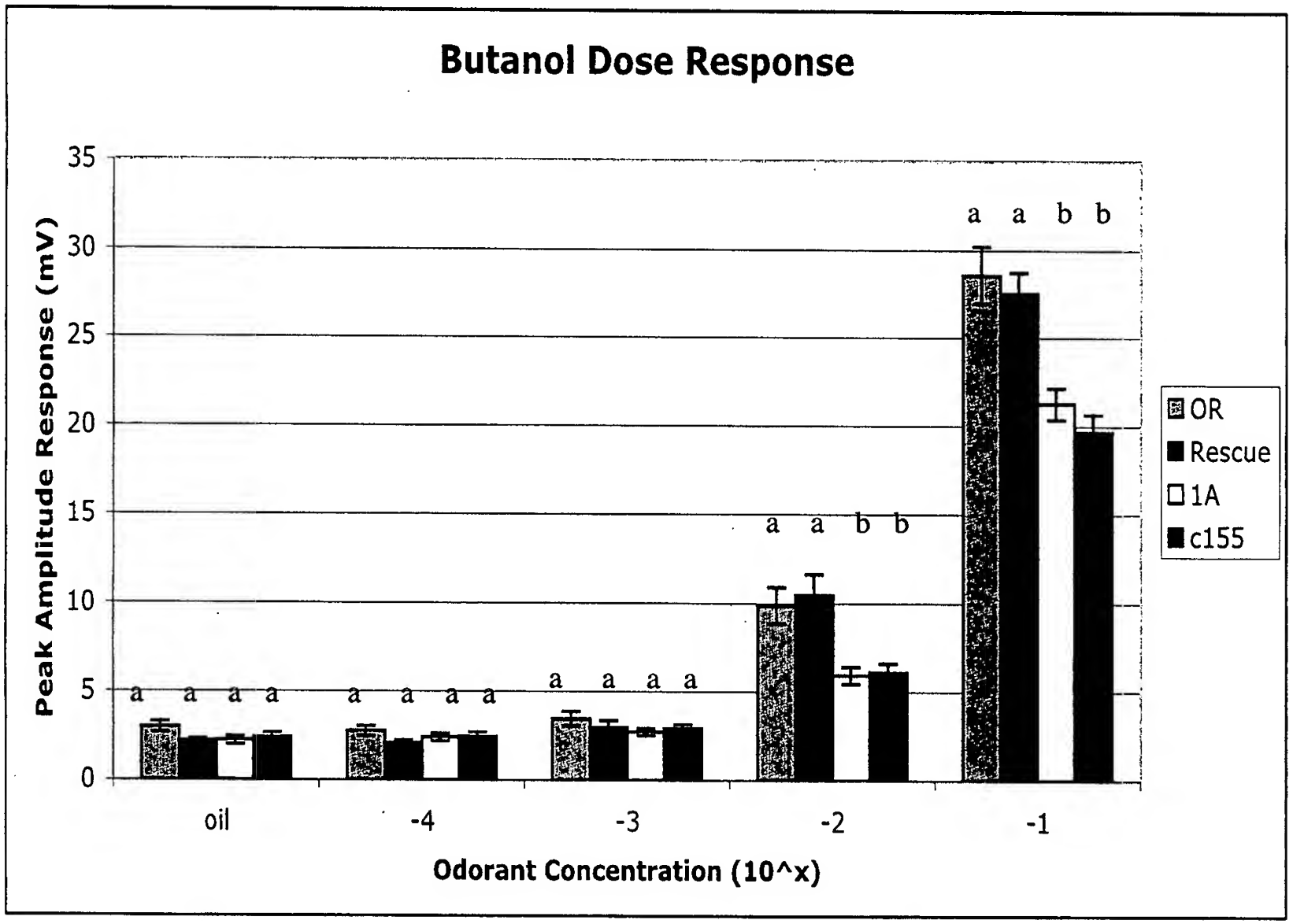
1A, n=11

c155, n=10

Error Bars represent S.E.M.

FIGURE 2

Butanol



Letters indicate statistically distinct

or

similar response groups

OR, n=15

Resuce, n=14

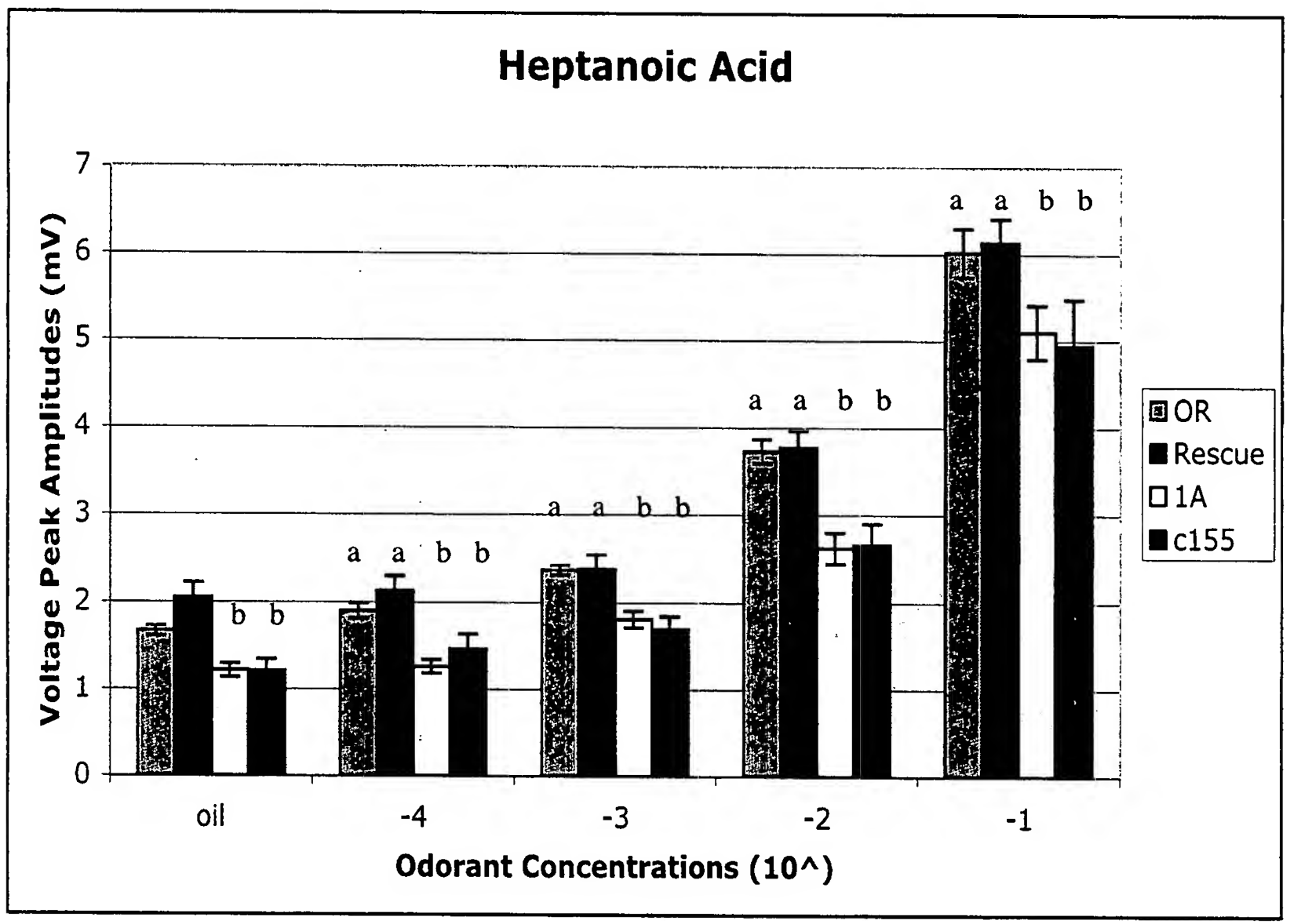
1A, n=15

c155, n=14

Error Bars represent S.E.M.

FIGURE 3

Heptanoic Acid



Letters indicate statistically distinct

or

similar response groups

OR, n=12

Resuce, n=15

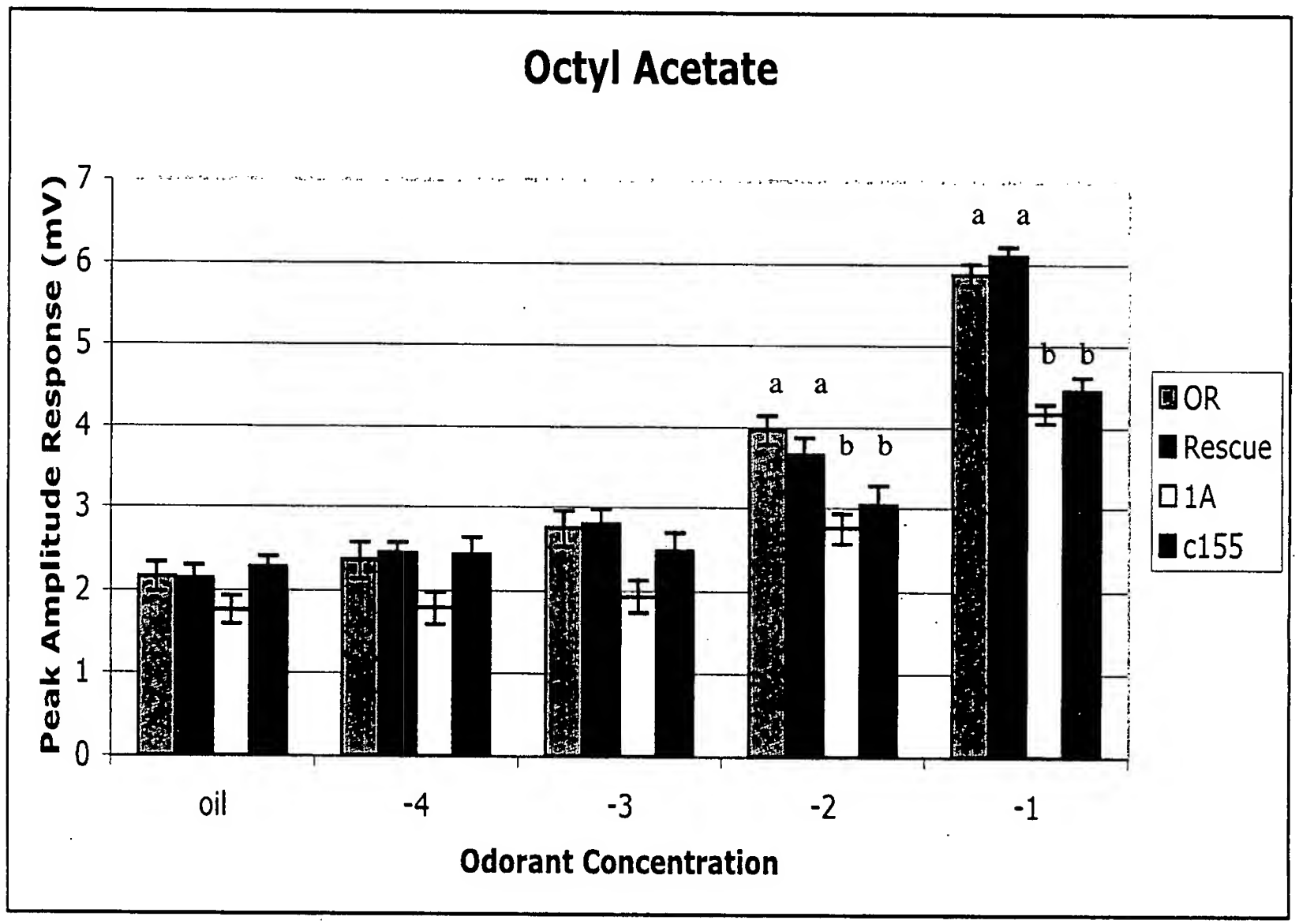
1A, n=10

c155, n=10

Error Bars represent S.E.M.

FIGURE 4

Octyl Acetate



Letters indicate statistically distinct

or

similar response groups

OR, n=12

Resuce, n=11

1A, n=11

c155, n=10

Error Bars represent S.E.M.

6. Referring now to Figure 5, there is shown results summarizing the effects of reducing AgArr1 levels on olfactory physiology in *Anopheles gambiae*. These experiments use RNA interference (RNAi) to induce gene silencing of AgArr1 expression coupled with electrophysiology in the form of electrolabellogram (ELG) analyses from the mosquito labellum to assess olfactory function. RNAi-based technologies has been developed in a number of invertebrate systems including *D. melanogaster* (Misquitta and Paterson, 1999) *C. elegans* (Fire et al., 1998), the vertebrate zebrafish (Li et al., 2000). The success of these experiments has encouraged wider application to mosquito species, most notably, *A. aegypti* (Adelman et al., 2002; Attardo et al., 2003; Sanchez-Vargas et al., 2004; Travanty et al., 2004), *Anopheles gambiae* (Blandin et al., 2002) and *An. stephensi* (Brown et al., 2003a; Brown et al., 2003b; Ito et al., 2002). It is my opinion that, taken together, loss-of-function reverse-genetic experiments show the role of olfactory genes in anophelines with regard to olfactory function and, importantly, the behaviors that are dependent on this critical sensory modality. The experiments performed herein were conducted as described by the Blandin et al., 2002 publication, a copy of which is attached hereto.

References cited in this paragraph

1. Adelman, Z. N., Sanchez-Vargas, I., Travanty, E. A., Carlson, J. O., Beaty, B. J., Blair, C. D., and Olson, K. E. (2002). RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. J Virol *76*, 12925-12933.

2. Attardo, G. M., Higgs, S., Klingler, K. A., Vanlandingham, D. L., and Raikhel, A. S. (2003). RNA interference-mediated knockdown of a GATA factor reveals a link to anautogeny in the mosquito Aedes aegypti. Proc Natl Acad Sci U S A *100*, 13374-13379.

3. Blandin, S., Moita, L. F., Kocher, T., Wilm, M., Kafatos, F. C., and Levashina, E. A. (2002). Reverse genetics in the mosquito Anopheles gambiae: targeted disruption of the Defensin gene. EMBO Rep *3*, 852-856.

4. Brown, A. E., Bugeon, L., Crisanti, A., and Catteruccia, F. (2003a). Stable and heritable gene silencing in the malaria vector Anopheles stephensi. Nucleic Acids Res *31*, e85.

5. Brown, A. E., Crisanti, A., and Catteruccia, F. (2003b). Comparative analysis of DNA vectors at mediating RNAi in Anopheles mosquito cells and larvae. J Exp Biol *206*, 1817-1823.

6. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

7. Ito, J., Ghosh, A., Moreira, L. A., Wimmer, E. A., and Jacobs-Lorena, M. (2002). Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. Nature *417*, 452-455.

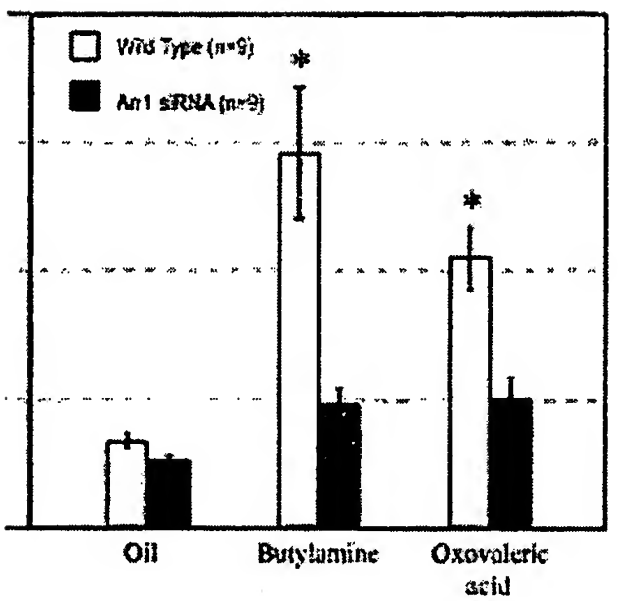
8. Li, Y.-X., Farrell, M. J., Liu, R., Mohanty, N., and Kirby, M. L. (2000). Double-Stranded RNA Injection Produces Null Phenotypes in Zebrafish. Dev Biol *217*, 394-405.

9. Misquitta, L., and Paterson, B. M. (1999). Targeted disruption of gene function in Drosophila by RNA interference (RNA-i): A role for nautilus in embryonic somatic muscle formation. Proc Natl Acad Sci U S A *96*, 1451-1456.

10. Sanchez-Vargas, I., Travanty, E. A., Keene, K. M., Franz, A. W., Beaty, B. J., Blair, C. D., and Olson, K. E. (2004). RNA interference, arthropod-borne viruses, and mosquitoes. Virus Res *102*, 65-74.

11. Travanty, E. A., Adelman, Z. N., Franz, A. W., Keene, K. M., Beaty, B. J., Blair, C. D., James, A. A., and Olson, K. E. (2004). Using RNA interference to develop dengue virus resistance in genetically modified Aedes aegypti. Insect Biochem Mol Biol *34*, 607-613.

FIGURE 5



RNAi mediated olfactory deficits in *An. gambiae*.
ELG responses were obtained from: wild type (non-injected) and RNAi injected mosquitoes. Mosquitoes were injected with 250nL of 10mM Ag-Arr1 siRNAs. Odorant concentration for all conditions is 1x10-2. "*" indicates varying degrees of significance: =p<0.01 (t-test).

7. As shown in Figure 5, it is my opinion that injection of AgArr1 siRNAs reproducibly resulted in significant reductions in olfactory physiology as assayed by ELG amplitudes in response to two representative odorants (10^{-2} dilution) that typically elicit strong ELG responses from wild-type and mock-injected control animals. Further experiments (data not shown) have utilized additional odorant stimuli as well as demonstrated that AgArr1 mRNA levels are also significantly reduced in response to target siRNAs. Furthermore, ELG amplitudes are similar to wild-type levels in mosquitoes that have been mock-injected with 1x injection buffer or with comparable amounts of siRNAs derived from non-insect gene targets. In my opinion, these data show that AgArr1 is involved in the olfaction cascade and olfactory physiology in *Anopheles gambiae* mosquitoes.

8. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Laurence J. Zwiebel

4/28/06
Date

Odorant-Specific Requirements for Arrestin Function in *Drosophila* Olfaction

C. Elaine Merrill,[1,2] Tracy M. Sherertz,[1] William B. Walker,[1,2] L. J. Zwiebel[1,2]

[1] Department of Biological Sciences, Vanderbilt University, 465 21st Avenue South, Nashville, Tennessee 37232

[2] Neuroscience Program, Vanderbilt University, 465 21st Avenue South, Nashville, Tennessee 37232

Received 4 May 2004; accepted 16 August 2004

ABSTRACT: **The ability to modulate olfactory sensitivity is necessary to detect chemical gradients and discriminate among a multitude of odor stimuli. Desensitization of odorant receptors has been postulated to occur when arrestins prevent the activation of downstream second messengers. A paucity of *in vivo* data on olfactory desensitization prompts use of *Drosophila melanogaster* genetics to investigate arrestins' role in regulating olfactory signaling pathways. Physiological analysis of peripheral olfactory sensitivity reveals decreased responsiveness to a host of chemically distinct odorants in flies deficient for *arrestin1* (*arr1*), *arrestin2* (*arr2*), or both. These phenotypes are manifest in odorant- and dose- dependent fashions. Additionally, mutants display altered adaptive properties under a prolonged exposure paradigm. Behaviorally, *arr1* mutants are impaired in olfactory-based orientation towards attractive odor sources. As the olfactory deficits vary according to chemical identity and concentration, they indicate that a spectrum of arrestin activity is essential for odor processing depending upon the particular olfactory pathway involved. Arrestin mutant phenotypes are hypothesized to be a consequence of down-regulation of olfactory signaling to avoid cellular excitotoxicity. Importantly, phenotypic rescue of olfactory defects in *arr1*[1] mutants is achieved through transgenic expression of wild-type *arr1*. Taken together, these data clearly indicate that arrestins are required in a stimulus-specific manner for wild type olfactory function and add another level of complexity to peripheral odor coding mechanisms that ultimately impact olfactory behavior.** © 2004 Wiley Periodicals, Inc. J Neurobiol 63: 15–28, 2005

Keywords: **arrestin; behavior; *Drosophila;* olfaction; physiology**

INTRODUCTION

Chemosensation drives a variety of behaviors across many species. Identification of G protein-coupled receptor (GPCR) families indicates that odor detection involves activation of second messengers that gate cationic channels (Firestein, 2001; Hill et al., 2002). However, considerably less is known about olfactory desensitization mechanisms.

Homologous GPCR desensitization occurs by concerted action of two classes of proteins: G protein receptor kinases (GRKs) and arrestins (Krupnick and Benovic, 1998). GRKs phosphorylate activated receptors to promote arrestin binding (Krupnick and Benovic, 1998). This interaction physically uncouples the receptor from G proteins to halt, or "arrest," the signaling cascade (Krupnick et al., 1997). Additionally, arrestins facilitate receptor recycling by promoting clathrin-mediated endocytosis (Goodman et al.,

Correspondence to: L. J. Zwiebel (l.zwiebel@vanderbilt.edu)
Contract grant sponsor: National Institutes of Health; contract grant numbers: DC5264 (C.E.M.) and DC04692/AI056402 (L.J.Z.)
This article includes Supplementary Material available via the Internet at http://www.interscience.wiley.com/jpages/0022-3034/suppmat

Published online 30 December 2004 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/neu.20113

1996; Luttrell et al., 1999; Laporte et al., 2000). Lastly, arrestins can activate alternative pathways such as MAP kinases to effect dramatic cellular responses including cytoskeletal reorganization, cellular proliferation, and differentiation (Luttrell et al., 1999; Pierce et al., 2001; Rakhit et al., 2001; Tohgo et al., 2002; Ge et al., 2003).

Arrestins have been well described in insect and mammalian visual systems (Wistow et al., 1986; Smith et al., 1990; Dolph et al., 1993). A separate subfamily, the "non-visual" arrestins, modulates many GPCRs including, among others, the adrenergic, opiod, and endothelin receptors (Luttrell and Lefkowitz, 2002). Recently, however, it has been suggested that categorizing arrestins as visual or non-visual is an oversimplification in insects (Merrill et al., 2002, 2003). Whereas the *Drosophila melanogaster* "visual" arrestin genes *arr1* and *arr2* clearly function in photoreceptor desensitization (Dolph et al., 1993), it now appears this is not their only role. Transcripts for *arr1* and *arr2* are detected in *Drosophila* olfactory organs, the antennae and maxillary palps (Merrill et al., 2002), and homologs of these genes in a lepidopteran, *Heliothis virescens*, an orthopteran, *Locusta migratoria*, and the dipteran mosquito *Anopheles gambiae* are similarly expressed in olfactory tissues (Raming et al., 1993; Merrill et al., 2003). These observations have led to the description of these genes as "sensory arrestins." Importantly, in a limited study, loss of sensory arrestin function correlates with impaired olfactory physiology in *Drosophila* (Merrill et al., 2002).

We now report a comprehensive evaluation of the *in vivo* physiological and behavioral roles of arrestins in olfactory function by analysis of arrestin mutants in the genetic model system *D. melanogaster*. We hypothesize that the loss of arrestin activity will have dramatic effects on olfactory sensitivity to a wide variety of odorant stimuli. The results indicate that sensory arrestins are required for olfactory responsiveness. Furthermore, the pattern of phenotypes associated with arrestin mutations outlines a spectrum of olfactory arrestin function depending upon the particular odorant pathway involved.

METHODS

Flies and Cultures

Oregon R (OR), obtained from Dr. C. Desai (Vanderbilt University), was used as the wild type control. Hypomorphic *arr1*^{1} *cn* and null *w; arr2*^{5} *ve h* mutant flies were a kind gift from Dr. P. Dolph (Dartmouth University) and have been extensively described (Dolph et al., 1993; Alloway and Dolph, 1999). Double mutant *arr1*^{1} *cn; arr2*^{5} *ve h* were also assayed. Flies were grown in plastic bottles on a standard cornmeal, molasses, agar, sugar medium supplemented with yeast and maintained at 18°C, 60% relative humidity, on a 12 h:12 h light:dark cycle.

Odorants

Eleven odorants were used in both the physiological and behavioral tests: 1-butanol, heptanal, ethyl acetate, and octyl acetate (Aldrich, Milwaukee, WI), and propanal, acetone, 2-heptanone, cyclohexanone, 1-octanol, proprionic acid, and heptanoic acid (Sigma, St. Louis, MO). Odorants were diluted in paraffin oil (Sigma, St. Louis, MO) to the concentrations indicated in figure legends.

Electroantennogram Physiology

The electroantennogram (EAG) responses of wild type and arrestin mutant flies were recorded similarly to previous reports (Alcorta, 1991; Riesgo-Escovar et al., 1994). In summary, 2–7-day-old adult female *Drosophila* were immobilized in the narrow end of a pipette tip such that only the anterior portion of the head protruded. Flies were placed 5 mm from the tip of a constant stream of clean humidified air provided by a stimulus control device (CS-05, Syntech, The Netherlands). Odorants were delivered by passing 0.5-s pulses of air through a glass Pasteur pipette containing a 1.5-cm diameter filter disk (VWR, West Chester, PA) saturated with 20 μl of diluted odorant into the constant air stream. The stimulus control device delivered a continuous flow rate of ~340 cc/min and a pulse rate of ~900 cc/min. The difference in air velocity between the continuous air stream and stimulus pulse did not produce a significant EAG response as evidenced by the fact that odor-free (oil-alone) responses are consistently low and reflect only minor background noise. Glass microelectrodes filled with 0.1M KCl transmitted electrical responses to odorant stimulation via silver/chloride wires to a signal acquisition system (IDAC232, Syntech, The Netherlands). Data was collected at 25 Hz, amplified 10× and converted from analog to digital, then displayed on a Gateway PC computer. EAG analysis was performed using EAG2000 software (Syntech, The Netherlands). The reference electrode was inserted into the head while the recording electrode was placed on the anterior dorso-medial surface of the third antennal segment in order to establish electrical contact. Three response parameters were examined in this analysis: amplitude, rise time, and fall time kinetics. The amplitude represents the peak voltage deflection in response to odorant presentation. Rise time and fall time kinetic parameters are measured as the time necessary to achieve 2/3 of the peak amplitude and the time taken to decay to 1/3 of the peak value, respectively (Alcorta, 1991). Lastly, an adaptive paradigm (Stortkuhl et al., 1999) was employed. In this experiment, flies are exposed to a desensitizing pulse of odorant (by directing the constant air stream through a bottle containing 1×10^{-1}

odorant for 1 min) and subsequently tested with 0.5-s pulses of 1×10^{-2} odorant at progressive time points after desensitization. The response amplitude was normalized relative to the naive response and plotted vs. time post-desensitization.

Larval Chemotaxis Assays

Chemotaxis assays were performed essentially as previously described (Heimbeck et al., 1999). Two 10-μl pipette tips (TipOne, USA Scientific, Ocala, FL), cut 12 mm in length, were placed on opposite sides of a Petri dish (85 mm diameter) containing 10 mL of 1% agar. A 6-mm filter disk (Schleicher and Schuell Microscience, Riviera Beach, FL) was secured on the pipette tip to ensure that odorants did not diffuse into the agar. Either 1.0 μl of diluted odorant or 1.0 μl of paraffin oil was pipetted onto the filter paper immediately before the assay began.

The agar plates were placed on a flat light source over a "baseball grid" consisting of two semicircular areas (radius 30 mm) designating the odorant and control regions (see Heimbeck et al., 1999). The placement of odor vs. control stimuli was switched randomly to eliminate visual cues.

Third instar feeding larvae were washed from fly food media over a handheld sieve with distilled water, placed in distilled water in small glass wells, and rinsed repeatedly to remove all visible food particles. Groups of 30–40 larvae were placed in the middle of the agar plate and gently spread along the central axis to allow independent movement. The plate was immediately covered and the response was recorded after 5 min.

To calculate the response, larvae inside the specified semicircles around odorant and control regions were counted, excluding all larvae in the central area (these larvae were considered "nonresponders"). A performance index (PI) was determined as in previous studies (Heimbeck et al., 1999) by the following equation: PI = (O−C)/(O+C), where O is the number of larvae that responded to the Odorant, and C is the number of larvae that responded to the oil Control. A minimum of 10 tests was performed for each concentration of each odorant for both wild type Oregon R and mutant $arr1^1$ larvae.

D. melanogaster Germline Transformation/Generation of Transgenic Rescue Lines

A DNA fragment encompassing the full-length coding sequence of wild type *arr1* was subcloned into the Drosophila *P* element vector, pUAST (Brand and Perrimon, 1993), for germline transformation of *D. melanogaster*. This vector was injected into pre-cellular embryos of true-breeding *yellow white*; *Kinked* $\Delta 2,3$ ($y^1\ w^{1118}$; *Ki* Δ *2,3* kindly provided by P. Kolodziej, Vanderbilt University) *D. melanogaster* (G0 generation). Individual w^+ G1 progeny laid by injected adults were crossed to *yw* (provided by P. Kolodziej, Vanderbilt University) to yield w^+ G2 flies that were in a *yw; Ki* $\Delta 2,3/+$ background. Male w^+ G2 flies were mated to *yw* females to eliminate the *Ki* $\Delta 2,3$ chromosome and establish true-breeding transgenic stocks.

Six independent transformed UAS-*arr1* lines were established in homozygous condition: UAS-$arr1^{D1C}$, UAS-$arr1^{D1G}$, UAS-$arr1^{D11}$ on the second chromosome and UAS-$arr1^{1C3B}$, UAS-$arr1^{1G5E}$, UAS-$arr1^{1I2A}$ on the third chromosome. In order to get the transgenic wild type *arr1* into an $arr1^1$ mutant background, third chromosome UAS insert lines were used (because $arr1^1$ is carried on the second chromosome). In each cross, the female parent bore the UAS transgene. Homozygous UAS lines were first crossed to the white-eyed, second chromosome balancer *GlaBcElp* (*w; In(2LR)Gla:* wg^{Gla-1} Bc^1 $Egfr^{E1}$ cn^1 bw^1 provided by C. Desai, Vanderbilt University), and transgenic progeny with small eyes (from the wg^{Gla-1} mutation) were selected. These G1 flies were crossed to homozygous *w;* $arr1^1$ *cn* mutants. G2 progeny from this cross were selected on the basis of the small-eye phenotype and for the presence of the transgene: *w;* $arr1^1$ *cn/GlaBcElp; p[UAS-arr1]/+* G2 adults were crossed to a linked second/third chromosomal balancer *w; SM6-TM6b* containing the aberration *T(2;3)SM6a-TM6B* (marked with al^2 Cy^1 dp^{hv1} cn^{2P} sp^2 $Antp^{Hu}$ e^1 Tb^1, from C. Desai, Vanderbilt University) in order to balance both the mutation and the transgene. The G3 progeny were selected based on the loss of the wg^{Gla-1} marker, and the presence of the transgene as well as the *Curly*-winged phenotype carried on the linked balancer. Finally, these G3 progeny carrying the balanced mutant allele and transgenic wild type *arr1* were self-crossed and their offspring lacking *Cy* were chosen and true bred into a homozygous *w;* $arr1^1$ *cn; p[UAS-arr1]* stock.

A similar genetic scheme was used to cross *w* $elavGal4^{c155}$ transgenic flies (a kind gift of M. Ramaswami, University of Arizona) expressing Gal4 under the neuronal *elav* promoter (Campos et al., 1987) into arrestin mutant backgrounds. Crossing female *w* $elavGal4^{c155}$; $arr1^1$ *cn* and male *w;* $arr1^1$ *cn; p[UAS-arr1]* generated progeny which express Gal4 and wild type *arr1* neuronally in an $arr1^1$ mutant background. Testing for transgenic rescue of olfactory phenotypes was performed on these progeny using the physiological and behavioral tests outlined above.

RESULTS

Arrestin Mutant Physiology

EAG Amplitude. These experiments were designed to test the hypothesis that arrestins are involved in the peripheral processing of different odor signals and may affect olfactory receptor neuron outputs. The maximum voltage deflection elicited by stimulation with 0.5-s pulses of 11 distinct odorants was measured from homozygotic flies carrying either the hypomorphic *arrestin1* allele, $arr1^1$; the null *arrestin2* mutation, $arr2^5$; or the double mutant, $arr1^1$, $arr2^5$ combination, which is deficient for both sensory ar-

restins. These mutations have been mapped and extensively characterized in the visual system of *D. melanogaster*, and the molecular nature of each allele has been well established (Dolph et al., 1993; Alloway and Dolph, 1999). $arr1^1$ has an intronic insertion of ~5 kb of DNA, limiting protein production to approximately 10% of wild type levels, whereas $arr2^5$ is an EMS-induced point mutation that causes a premature stop codon at amino acid 20 and eliminates detectable ARR2 protein (Dolph et al., 1993; Alloway and Dolph, 1999). We chose a range of 11 different odorants to span a variety of chemical groups and carbon chain lengths, as these features have been implicated in odorant receptor activation (Araneda et al., 2000). The amplitude responses to each of these individually applied odorants were compared between the wild type Oregon R strain and arrestin mutant alleles across a range of concentrations. Whereas distinct odorants evoke heterogenous response amplitudes, significant differences exist between wild type and arrestin mutant flies for every odorant tested; the peak response for arrestin mutants is consistently lower than that of wild type (Figs. 1 and 2, Supplementary Table 1). However, the amplitude reduction in arrestin mutant flies is influenced by odorant identity and concentration. Overall, arrestin mutant phenotypes designate classes of odorant signaling pathways that require varying degrees of arrestin function (Figs. 1 and 2, Supplementary Table 1).

High Arrestin Requirement. Odorants in Class I have a critical arrestin requirement such that any arrestin deficit decreases the amplitude 25–50% across all odorant concentrations. For example, stimulation with increasing concentrations of heptanone elicits a range of amplitudes from 4.5–23.7 mV for wild type flies, in contrast to only ~3–15 mV for arrestin mutants [Fig. 1(A)]. The $arr1^1$ allele shows a trend towards a more severe phenotype than the *arr2* mutant, suggesting that it could play a more dominant role in this pathway [Fig. 1(A)], however, this observation requires more rigorous testing to confirm the specific roles of *arr1* vs. *arr2* in response to heptanone. Furthermore, it is impossible to determine the exact contributions of the two genes as $arr1^1$ is hypomorphic, and $arr2^5$ is a null allele. As with heptanone, arrestin mutant response amplitudes to octanol and propanal are reduced relative to wild type at all concentrations [Fig. 1(A)]. The sensitivity of each of these dose-response profiles to the loss of either sensory arrestin indicates that both are required for modulating receptor signaling cascades activated by Class I odorants.

Class II includes odorant pathways that are also highly sensitive to the loss of either sensory arrestin, although principally when higher concentrations of odorant are presented. At the highest concentration of butanol, loss of either *arr1* or *arr2* yields amplitudes of 21–24 mV, an 18–27% reduction relative to wild type. Interestingly, the double mutant exhibits only an 18.4 mV response, 37% decreased relative to wild type. This pattern is unique in Class II and suggests that both sensory arrestins are required, and their combined loss further impinges on olfactory responsiveness to this odorant [Fig. 1(B)]. At high concentrations of heptanoic acid and proprionic acid, mutations in either sensory arrestin gene cause a ~30–40% reduction in amplitude relative to wild type, implying that both sensory arrestins function in the response to carboxylic acids. [Fig. 1(B)]. Responses to high doses of the ester octyl acetate are also decreased (~20–25%) in arrestin mutants [Fig. 1(B)]. Overall, these outcomes suggest that sensitivity to Class II odorants requires arrestin function at higher concentrations such that loss of either gene causes impaired signaling. Thus, the olfactory system can maintain wild type function up to a certain stimulation level at which arrestin activity becomes critical.

Low Arrestin Requirement. Unlike Classes I and II in which both arrestins are required, odorants in Classes III and IV convey a different requirement for arrestin function (Fig. 2). Sensory arrestin activity in signaling pathways activated by the Class III odorants acetone and heptanal appears functionally redundant because either single arrestin mutant is unaffected, however, the loss of both genes leads to an impaired response. Following stimulation with acetone or heptanal, the double mutant, $arr1^1$, $arr2^5$, produces amplitudes that are ~60% reduced relative to wild type across all concentrations [Fig. 2(A)]. For example, at a 1×10^{-2} dilution of heptanal, wild type, $arr1^1$, and $arr2^5$ demonstrate responses of ~16.4 mV compared to the double mutant amplitude of 11.0 mV [Fig. 2(A)]. The conclusion from these results is that arrestin activity is redundant in these pathways, and signaling becomes disrupted only when both genes are compromised.

When cyclohexanone and ethyl acetate, which comprise Class IV, are tested, a slight decrease in amplitude is observed only at the highest odorant concentration [Fig. 2(B)]. In the case of cyclohexanone, $arr1^1$ mutations cause a 15% reduction in response amplitude relative to wild type (19.8 mV vs. 23.9 mV); *arr2* mutants display similarly depressed amplitudes in response to high doses of ethyl acetate [Fig. 2(B)]. In summary, these Class IV odorants

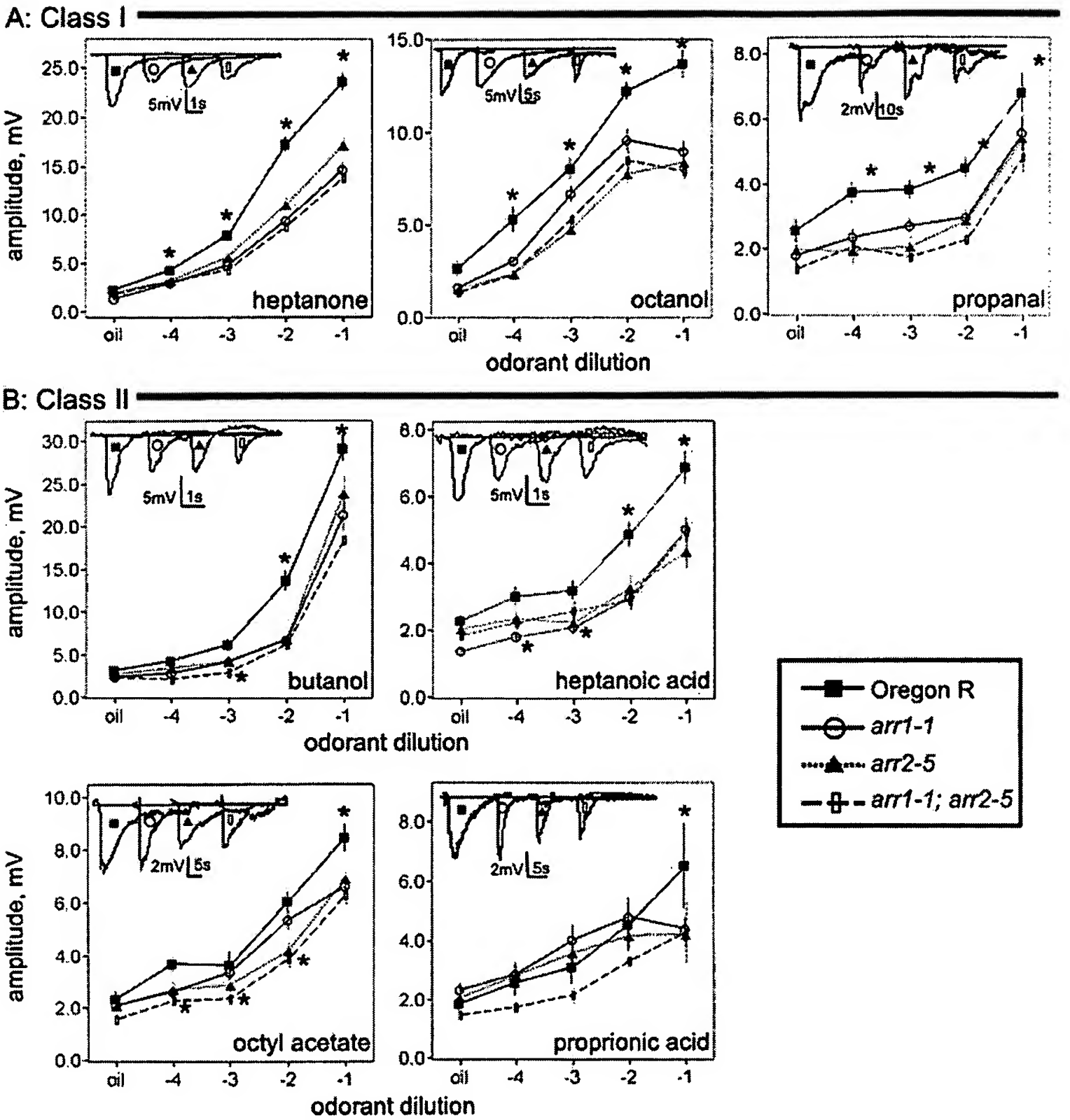


Figure 1 Class I and Class II odorants define signaling pathways with a high requirement for arrestin function. EAG dose-responses to a variety of odorants were obtained from wild type Oregon R, single sensory arrestin mutants or the double mutant (closed squares, wild type; open circles, $arr1^1$; closed triangles, $arr2^5$; open rectangles, $arr1^1$; $arr2^5$). Amplitude is measured as the peak voltage deflection (in mV) in response to stimulation with 0.5-s pulses of odorants. Odorant concentration is presented as a 1×10^x dilution in mineral oil. (A) Heptanone, octanol, and propanal (top panel) define Class I odorants which have a strict requirement for arrestin function such that loss of any sensory arrestin causes reduced EAG responses at all concentrations of odorant. (B) Butanol, heptanoic acid, octyl acetate, and proprionic acid (lower panels) constitute Class II odorants. These pathways also have a high requirement for arrestin function as loss of either sensory arrestin impairs EAG amplitudes, however, these effects are only observed at high odor concentrations. * = $p < 0.05$ different from wild type. An "*" located above a particular dilution indicates all arrestin mutants are different from wild type, whereas an "*" placed beside a particular point indicates only this genotype is different from wild type. Error bars indicate S.E.M.; n = 7–20; insets: representative raw EAG responses to 1×10^{-2} odorant for each genotype (denoted by the corresponding symbol).

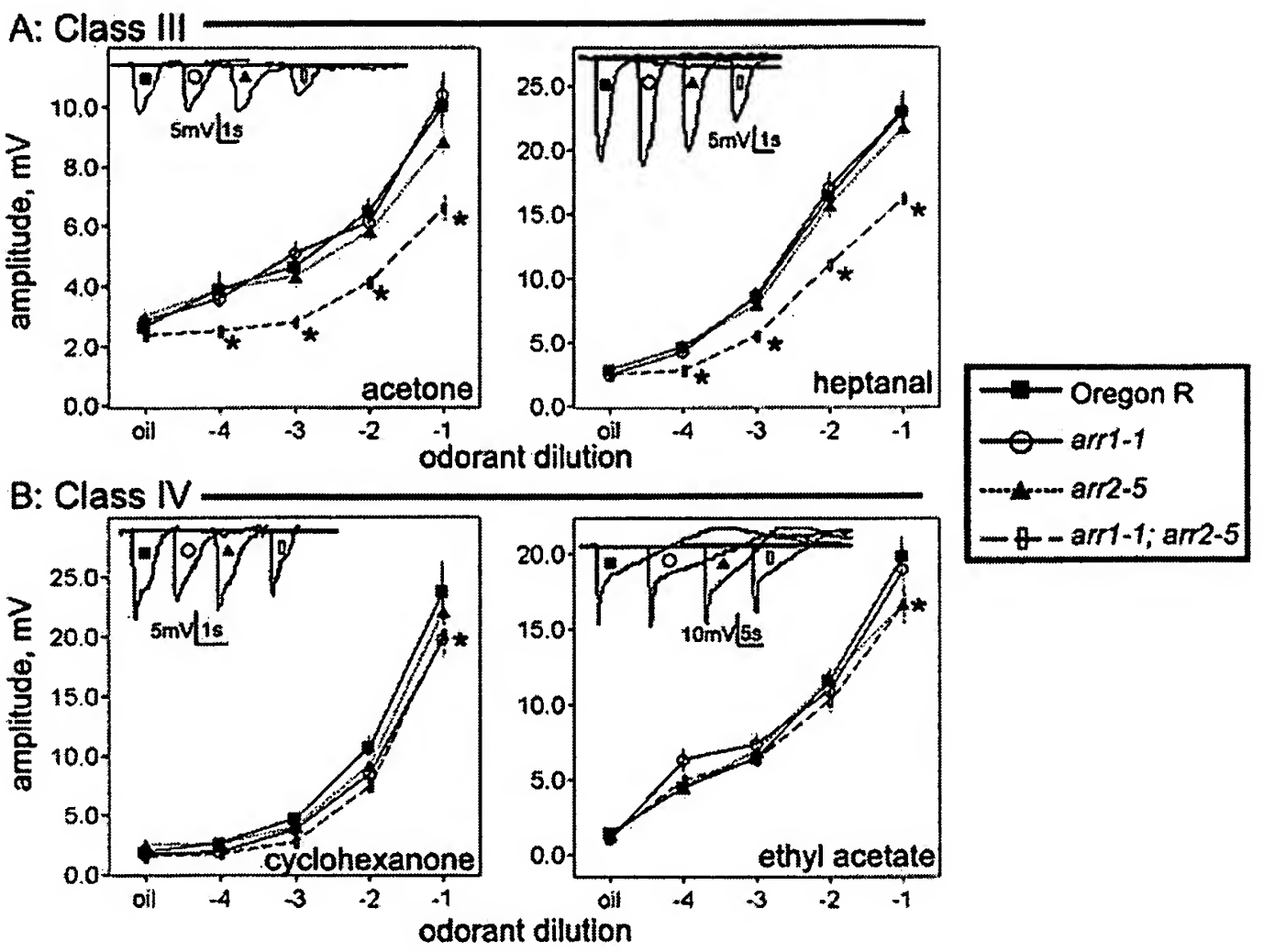


Figure 2 Class III and Class IV signaling pathways demonstrate a low requirement for arrestin activity. EAG dose-responses were recorded from wild type and sensory arrestin mutant *D. melanogaster* (closed squares, wild type; open circles, $arr1^1$; closed triangles, $arr2^5$; open rectangles, $arr1^1$; $arr2^5$) and are presented as in Figure 1. (A) Stimulation with Class III odorants, acetone and heptanal (top panel), results in normal EAG amplitudes from single sensory arrestin alleles, but exhibits decreased responses at all concentrations in double arrestin mutant flies, indicating that sensory arrestin function is redundant in these pathways. (B) Class IV odorants ethyl acetate and cyclohexanone (lower panel) show a minimal requirement for arrestin function. Decreased EAG amplitudes are manifested in either $arr1^1$ or $arr2^5$ and the double mutant (depending on the odorant), and only at the highest odorant intensity. * = $p < 0.05$ different from wild type. When "*" is located above a particular dilution, all arrestin mutants are different from wild type, and when "*" is placed beside a particular point, only this genotype is different from wild type. Error bars indicate S.E.M.; n = 5–20; insets: representative raw EAG responses to 1×10^{-2} odorant for each genotype (denoted by the corresponding symbol).

exhibit mild requirements for a particular arrestin, but only at the highest stimulus intensity.

Amplitude analysis reveals that strength of the response to certain odorants (i.e., Class I and Class II) is dependent upon function of both sensory arrestins. In other signaling pathways (i.e., Class III odorants), sensory arrestins are redundant, such that a loss of responsiveness is only observed when both arrestins are deficient. Lastly, the olfactory response to particular odorants (i.e., Class II and Class IV) is largely unaffected until the stimulus intensity has reached a level at which arrestin activity becomes critical. Considering the various arrestin mutant effects on amplitude for odorant Classes I–IV, it is apparent that a spectrum of arrestin function exists for different odorant signaling pathways.

EAG Kinetics. The kinetic parameters of odorant responses were also calculated from the EAGs using standard measures (Alcorta, 1991). The rise time of the response to various odorants ranges from ~0.15–0.43 s, however, no considerable significant differences are detected between wild type and arrestin mutant flies for any odorant tested [e.g., propanal; Fig. 3(A)]. The fall time of the response is more

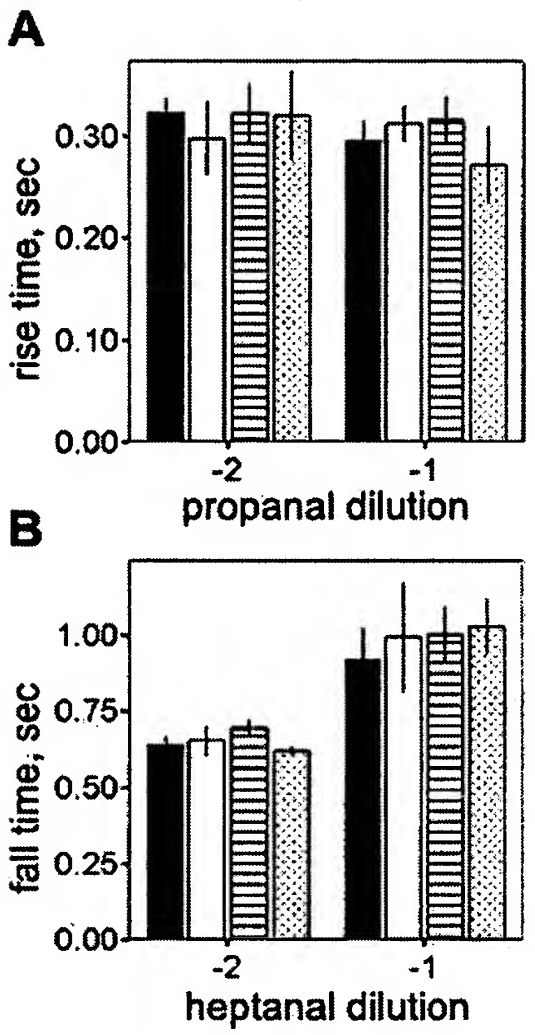


Figure 3 Kinetic analysis of EAG responses does not reveal differences between wild type and arrestin mutant *Drosophila*. (A) Rise time kinetics in response to stimulation with propanal are indistinguishable across all genotypes. This parameter is highly consistent and is not greatly influenced by concentration. (B) In response to heptanal, fall time is similarly unaffected by the loss of arrestins. Fall time kinetics are increased as the odor dose increases in intensity. Black bars, wild type Oregon R; white bars, $arr1^1$; striped bars, $arr2^5$; stippled bars, $arr1^1$; $arr2^5$. Odorant concentration is presented as a 1×10^x dilution in mineral oil. Error bars indicate S.E.M.; $n = 5–14$.

variable depending on the odorant tested, ranging from 0.5 s to a full 10 s. However, once again, we did not uncover any consistent significant differences between wild type and arrestin mutants in analysis of fall time kinetics [e.g., heptanal; Fig. 3(B)].

EAG Adaptation. A desensitization paradigm was utilized to detect differences in the ability of wild type or arrestin mutants to desensitize to prolonged odorant exposure and recover from this adaptation (Stortkuhl et al., 1999). Following a naive test pulse of 0.5 s of 1×10^{-2} odorant, flies were exposed to a 1-min desensitizing stimulus of a 1×10^{-1} concentration of odorant and then tested at progressive intervals afterwards with 0.5-s pulses. Post-desensitization responses were normalized to naive response in order to eliminate amplitude effects.

This paradigm uncovered minor differences in the initial desensitization, with little effect on recovery from adaptation; however, as with the amplitude analysis, these differences varied depending on the odor presented. When desensitized with the Class I odorant heptanone, wild type responses are decreased to 60% of naive levels. The arrestin mutants have a slightly altered initial desensitization: $arr1^1$ exhibit a less desensitized response than wild type (~70% of naive), desensitization of $arr2^5$ responses is slightly enhanced (only ~40% of naive), and the double mutant displays an intermediate phenotype [Fig. 4(A)]. The desensitization to, and recovery from, prolonged exposure to butanol was not significantly different across all genotypes [Fig. 4(B)]. In response to heptanal, a Class III odorant, the initial desensitization is similar among all genotypes. However, $arr2^5$ flies are marginally delayed in the recovery to this odorant relative to wild type [Fig. 4(C)]; such a lag may be observed due to the lower initial value of $arr2^5$ responses. When tested with the Class IV odor ethyl acetate, all mutants have a higher relative response at 0 and 0.5 s post-desensitization. Whereas wild type flies lose 60% of their signaling capability, arrestin mutants are only decreased by ~20–40%, suggesting that the loss of sensory arrestins impairs the ability to desensitize to prolonged odorant stimulation [Fig. 4(D)]. Overall, these results reveal that disrupting arrestin function can disrupt desensitization, suggesting a need for arrestins to temper receptor sensitivity upon protracted exposure. As these phenotypes again display odorant-specific alterations, the results further support the hypothesis that different signaling cascades have diverse requirements for arrestin activity.

Arrestin Mutant Behavior

A well-characterized larval chemotaxis assay (Heimbeck et al., 1999) was employed to examine the olfactory behavioral outputs of $arr1^1$ mutant *Drosophila* relative to wild type. Because *arr2* mutants have sluggish movements (data not shown), they and the double mutant were excluded from this locomotor-based analysis to prevent confounding results due to decreased mobility.

As previously described, 30–40 third instar larvae are placed in an agar arena containing an odor source opposed to a control site; after 5 min, the number of larvae responding to both locations is counted and used to determine a performance index (PI) (Heimbeck et al., 1999). Positive PIs indicate attractive responses (+1 = complete attraction), whereas neg-

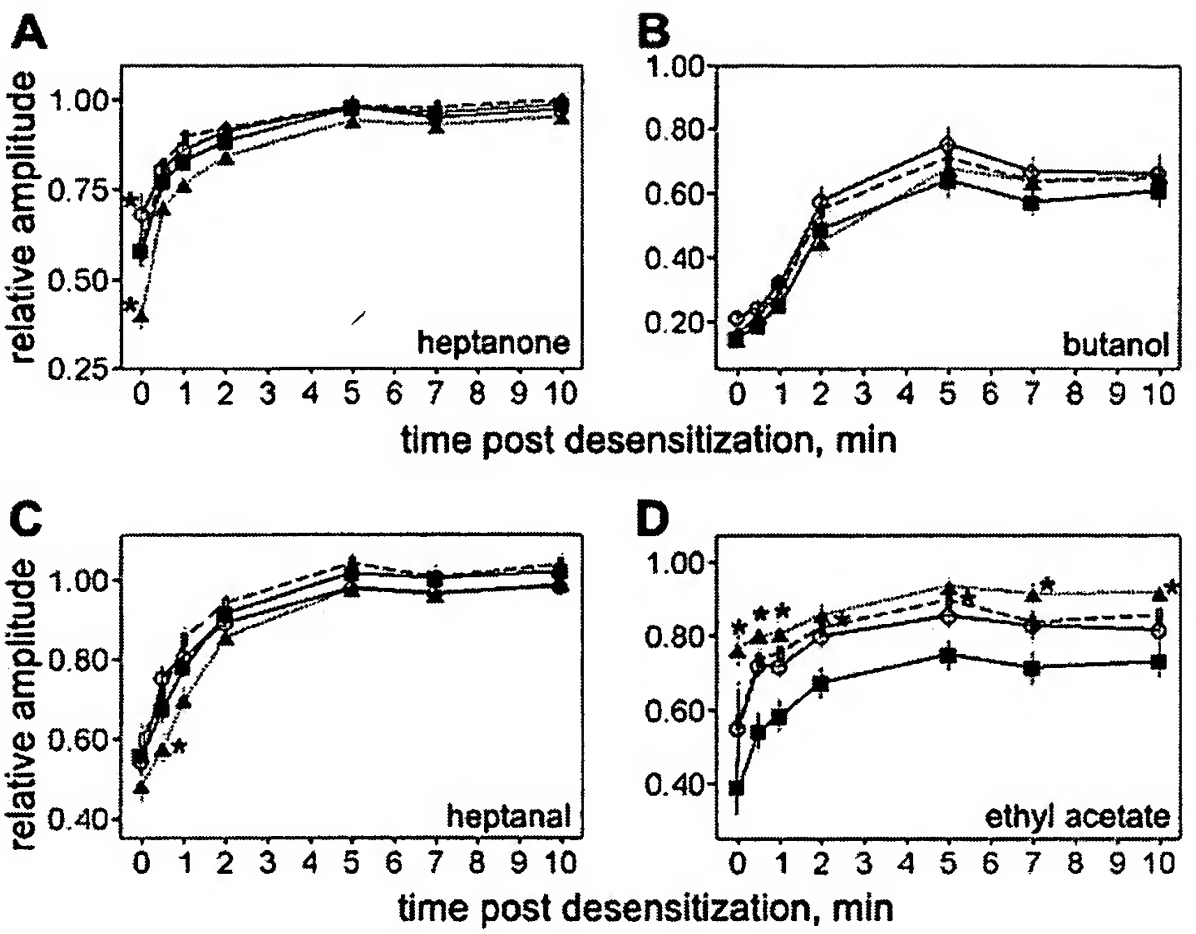


Figure 4 Prolonged odorant exposure reveals altered desensitization and recovery in arrestin mutant flies. (A) Initial desensitization to extended stimulation with the Class I odorant heptanone shows defective desensitization by *arr1*^{1} mutants but enhanced desensitization in *arr2*^{5} flies. The double mutant allele is intermediate of these phenotypes. (B) Desensitization to and recovery from a prolonged exposure of the Class II odorant butanol is similar in all genotypes, however, arrestin mutant flies show a trend towards faster recovery. (C) Prolonged exposure to the Class III odorant heptanal results in comparable desensitization for each genotype, however, *arr2*^{5} mutants are slightly retarded in their recovery. (D) Each sensory arrestin mutant displays defective desensitization in result to an adaptation with the Class IV odorant ethyl acetate. Closed squares, wild type; open circles, *arr1*^{1}; closed triangles, *arr2*^{5}; open rectangles, *arr1*^{1}; *arr2*^{5}. * = $p < 0.05$ different from wild type. When "*" is located above a particular time point, all arrestin mutants are different from wild type, and when "*" is placed beside a particular point, only this genotype is different from wild type. Error bars indicate S.E.M.; n = 10–15.

ative values reflect repulsive reactions (−1 = complete repulsion); a PI of 0 suggests neutral or indifferent behavior.

In response to the 11 odorants tested, *arr1*^{1} mutants predominantly display decreased attraction relative to wild type (Fig. 5). At all concentrations of Class I odorants heptanone and octanol, and Class II odorants butanol and proprionic acid, *arr1*^{1} mutants react with stunted behavioral responses that are 20–50% lower than wild type [Fig. 5(A)]. To provide one example, wild type flies demonstrate attraction towards increasing concentrations of proprionic acid with PIs ranging from 0.55–0.75, yet *arr1*^{1} PIs only reach 0.2–0.3 [Fig. 5(A)]. Such a severe reduction in chemotactic behavior suggests that *arr1* is critically important for these olfactory-mediated behaviors.

Attraction to the remaining Class II and both Class III odorants is indistinguishable between wild type and *arr1*^{1} larvae at lower concentrations, but the *arr1*^{1} mutant again reveals a decreased behavioral output when higher concentrations of odorant are tested [Fig. 5(B)]. Wild type *Drosophila* are mildly attracted to higher concentrations of heptanoic acid and octyl acetate, yet *arr1* mutants are nearly insensitive to these odorants [Fig. 5(B)]. Both genotypes show indifference to most concentrations of the Class III odorant acetone, but whereas wild type react positively at the highest concentration, *arr1*^{1} mutants actually exhibit a repulsive behavioral response (PI −0.09) [Fig. 5(B)]. As with acetone, increasing concentrations of heptanal cause a repulsive effect on *arr1*^{1} mutants [Fig. 5(B)]. Behavioral responses to

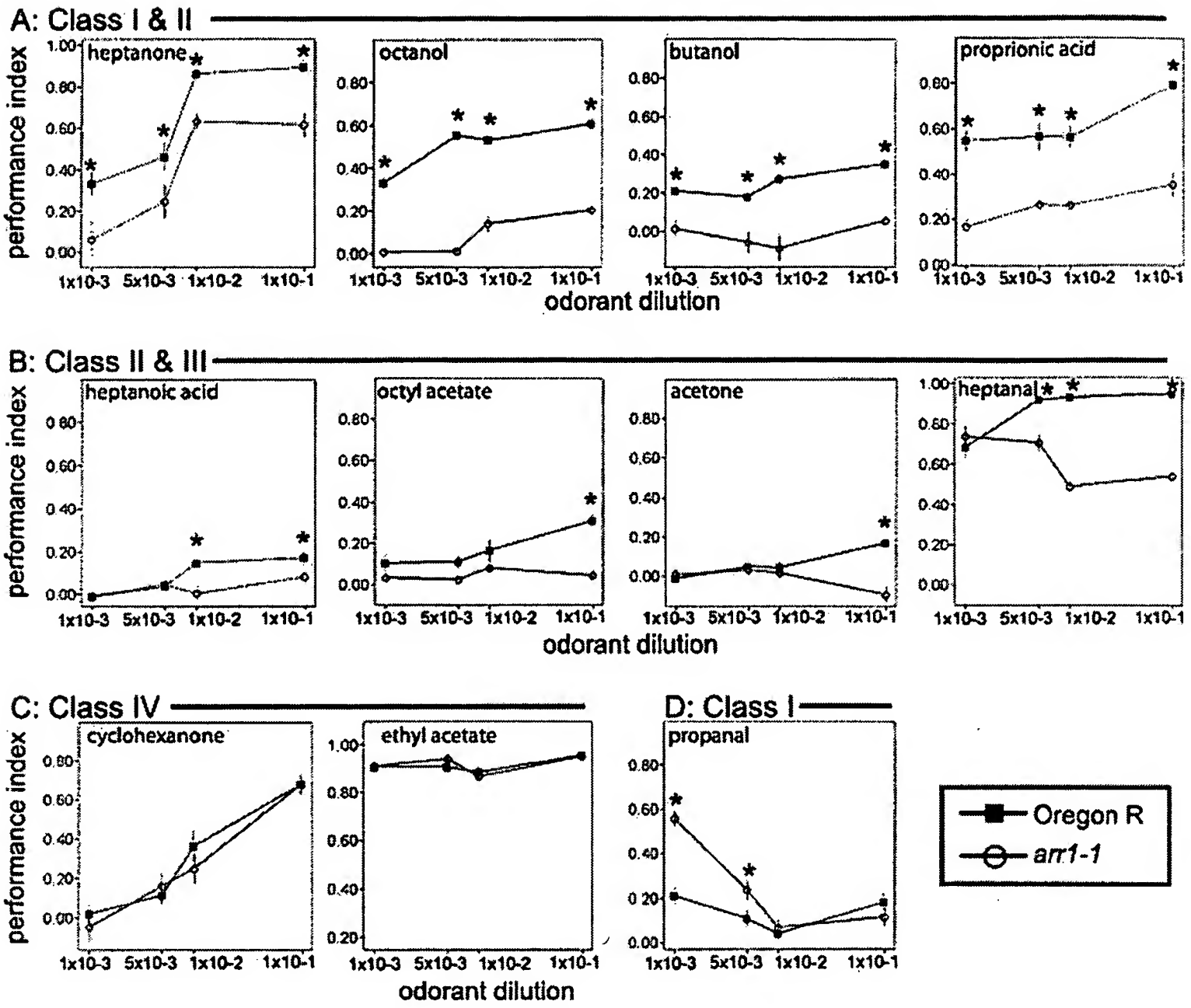


Figure 5 Larval chemotaxis assays demonstrate a requirement for *arr1* function in larval olfactory behavior. (A) *arr1* mutants have decreased attraction across all concentrations of Class I odorants heptanone and octanol, and Class II odorants butanol and heptanoic acid. (B) In response to Class II odorants octyl acetate and proprionic acid and both Class III odorants (acetone and heptanal), *arr1* mutant *Drosophila* demonstrate impaired orientation to the odor source, but only at higher concentrations. (C) Wild type olfactory behavioral responses to Class IV odorants cyclohexanone and ethyl acetate are observed in the presence of the $arr1^{1}$ allele. (D) The Class I odorant propanal elicits minimal attraction from wild type flies, but enhanced responsiveness from the $arr1^{1}$ at low odorant doses but indistinguishable responses at higher concentrations. Closed squares, wild type; open circles, $arr1^{1}$; * = $p < 0.05$ different from wild type; error bars indicate S.E.M.; $n = 20$.

odorants in Class IV, cyclohexanone and ethyl acetate, do not distinguish any significant differences between wild type and arrestin mutant larvae [Fig. 5(C)]. Thus, as with the amplitude analysis, behavioral responses to these odorants do not rely heavily on arrestin function.

Remarkably, the Class I odorant propanal shows a unique behavioral dose-response pattern [Fig. 5(D)] that is essentially the reverse of what is observed in every other case. Attraction typically increases concomitantly with increases in stimulus intensity, however, propanal appears inversely attractive relative to concentration. Moreover, responses to all other odorants show $arr1^{1}$ to have stunted performance, yet they are more attracted than wild type to low doses of this odorant. This pattern could reflect some developmental or experience-dependent alteration in the signaling cascade due to the arrestin mutation that has not occurred in the same manner as with other odorant pathways.

In general, the behavioral phenotypes approximate those seen in amplitude analysis: more severe deficits in arrestin mutant responses to Class I/II odorants, somewhat less severe for Class III, and little-to-no alteration in response to Class IV odorants. These findings are consistent with the idea that sensory arrestin activity follows a continuum depending upon particular odorant pathways.

Transgenic Rescue by Wild Type *arr1*

In an effort to firmly establish the role of arrestins in olfactory processes, we carried out phenotypic rescue experiments via germline transformation (Rubin and Spradling, 1982). In these studies, we took advantage of the bipartite Gal4/UAS system in order to restore expression of a wild type copy of the *arr1* gene in all olfactory and non-olfactory neurons of $arr1^1$ mutant flies using the *elav*Gal4 driver (Campos et al., 1987). In these transgenic flies, we observe EAG response amplitudes to heptanone and butanol at levels that are indistinguishable from wild type Oregon R control flies [Fig. 6(A, B)]. Importantly, decreased EAG amplitudes are consistently observed in all genetic backgrounds that preclude neuronal expression of the *arr1* transgene (Gal4 and UAS mutant lines individually). Furthermore, larval olfactory behavioral responses to octanol are restored to wild type levels in transgenic animals expressing neuronal *arr1*, whereas all larvae bearing the $arr1^1$ allele with only Gal4 or UAS still exhibit significant reductions in attraction to this odorant [Fig. 6(C)]. These data, as well as similar results for additional odorants (data not shown), firmly establish the specific role of arrestin expression in olfactory responses.

DISCUSSION

The immediate findings of these studies support the hypothesis that arrestins are crucial for olfactory system function. The first method of inquiry into the role of arrestins in insect olfaction was a physiological measure of peripheral olfactory responsiveness using electroantennograms. Importantly, arrestin mutant flies demonstrate decreased amplitude relative to wild type; however, these phenotypes are variably manifested depending upon the odorant and concentration tested (Figs. 1 and 2, Supplementary Table 1). Class I and II odorants rely on the function of both arrestins. It has been shown that vertebrate β-arrestins are capable of interacting with multiple receptor types (Krupnik and Benovic 1998), raising the possibility that both sensory arrestins may be interacting with an identical type of receptor. Alternatively, the sensory arrestins may function on a cellular level, with each arrestin regulating receptors from independent olfactory neurons that overlap in odorant sensitivity. Single-unit recordings may resolve these possibilities. Class III odorants also require arrestin function, however, in response to these stimuli, arrestins act redundantly as only double mutations reveal a physiological defect. Lastly, Class IV odorants do not appear to have a strong requirement for sensory arrestins.

These results are consistent with prior observations of arrestin function in peripheral physiology that mapped olfactory phenotypes to the sensory arrestin loci (Merrill et al., 2002). This study provides a more comprehensive examination of the role of arrestins in distinct olfactory responses and proposes a spectrum of arrestin requirements that depends upon the receptor pathways activated by a particular odorant (i.e., Classes I–IV). Whereas there are no molecular features that link the various odorants within a class, the possibility exists that the groupings reflect the type of odorant receptor or underlying second messenger system activated by these odorants. Arrestins thus contribute another level of complexity in peripheral olfactory coding, independent of combinatorial receptor stimulation and other, higher-order (antennal lobe) glomerular activation and integration (Firestein, 2001).

Kinetic analysis of EAGs did not uncover any disparity in rise time or fall time of the response between wild type and arrestin mutants (Fig. 3). This result is somewhat surprising, as mutations in these genes affect visual response kinetics (Dolph et al., 1993). Whereas individual $arr1^1$ alleles have no effect, a null mutation of *arr2*, $arr2^3$ extends the decay phase of visual responses, and double mutants show severely prolonged decay. Furthermore, whereas the loss of arrestins induces significant light-dependent photoreceptor apoptosis (Dolph et al., 1993; Alloway and Dolph, 1999), similar degeneration is not observed in the olfactory system (Merrill et al., 2002). These results may indicate that arrestin function is not required for proper physiological kinetics, yet it is likely that the inability to detect differences stems from the nature of the EAG. As these recordings reflect the combined activity of a diverse population of olfactory receptors, it is possible that subtle kinetic discrepancies in restricted subsets of cells may be missed due to summation of many response profiles. Single-sensillum recordings may better address this analysis, however, limiting the response to one particular sensilla/receptor would necessarily affect the overall scope of receptor-mediated activity.

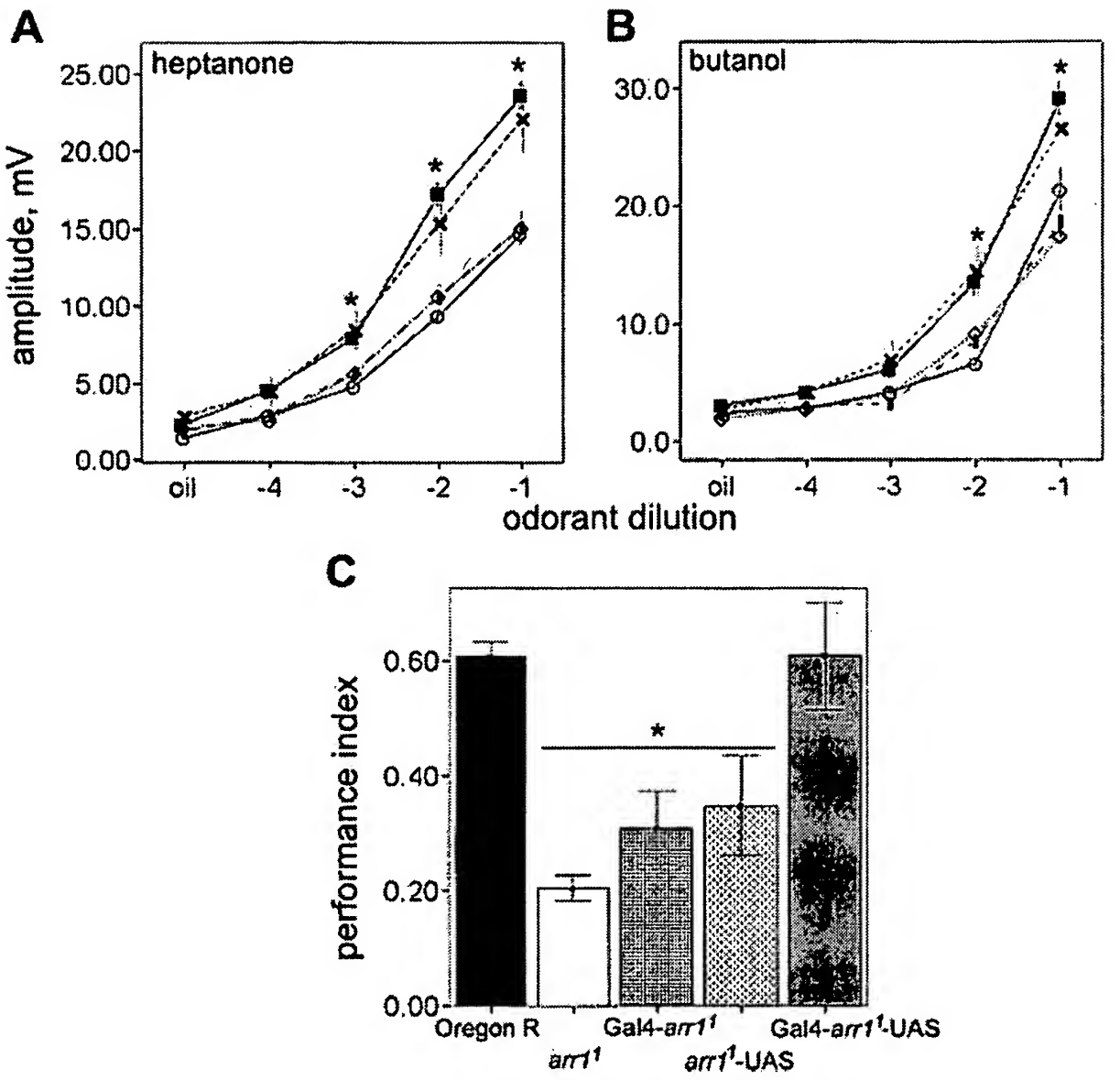


Figure 6 Transgenic expression of *arr1* rescues olfactory phenotypes of arrestin mutant *Drosophila*. (A) Whereas the *elavGal4^{c155}* pan-neural driver and transgenic UAS-*arr1^{112A}* in *arr1^{1}* mutant background retain reduced amplitude responses to stimulation with increasing concentrations of heptanone, combinations of the *elavGal4^{c155}* driver and the *arr1^{112A}* UAS transgenes results in a rescued, wild type dose-response profile. (B) Both Gal4 and UAS lines independently display the decreased amplitude response to high concentrations of butanol due to the *arr1* mutant background. However, expression of transgenic *arr1^{112A}* in neurons by the *elavGal4^{c155}* driver rescues the amplitude deficit. (A, B) Wild type and *arr1^{1}* data reproduced from Figure 1. Closed squares, wild type; open circles, *arr1^{1}*; open diamonds, *elavGal4^{c155}*, *arr1^{1}*; closed rectangles, *arr1^{1}*, *UAS-arr1^{112A}*; closed "×"; *elavGal4^{c155}*, *arr1^{1}*, *UAS-arr1^{112A}*. $n = 3$–10; * = $p < 0.05$ arrestin mutant lines different from wild type and rescue line. (C) Transgenic expression of wild type *UAS-arr1^{112A}* rescues olfactory behavioral responses to 1×10^{-1} octanol. *arr1^{1}* mutants, including those carrying either the *elavGal4^{c155}* or UAS-*arr1^{112A}* transgenes have diminished attraction to octanol, yet combinations of these alleles drive neuronal expression of *arr1* in a homozygous *arr1^{1}* background to restore behavioral response to wild type levels. Wild type and *arr1^{1}* data reproduced from Figure 1. Black bar, wild type (Oregon R); white bar, *arr1^{1}*(*arr1*). Crossed bar, *elavGal4^{c155}*; *arr1^{1}* (Gal4-*arr1^{1}*). Hatched bar, *arr1^{1}*, *UAS-arr1^{112A}* (*arr1^{1}*-UAS). Gray bar, *elavGal4^{c155}*; *arr1^{1}*, *UAS-arr1^{112A}* (Gal4-*arr1^{1}*-UAS). $n = 3$–20; * = $p < 0.05$ arrestin mutant lines different from wild type and rescue line.

An adaptive paradigm was employed to test responses after prolonged stimulus exposure. Again, these results varied depending on the odorant tested and confirm the disparity of arrestin function in olfactory signaling cascades corresponding to diverse olfactory receptor populations. One potential rationale for the mild effects is that olfactory sensory neurons have become down-regulated as a result of the loss of arrestins, and the observed desensitization occurs is due to the action of other mechanisms. Heterologous

nonspecific receptor desensitization can occur via phosphorylation by PKA or PKC, GTPase activating proteins can inactivate G proteins, phosphodiesterases can cleave second messengers, and channel phosphorylation can decrease conductance (Luttrell and Lefkowitz, 2002; Tan et al., 2003). However, as Class IV odorants are unaffected by arrestin mutations in terms of amplitude, it appears they have not downregulated their signaling potential. For this reason, upon prolonged stimulation, the requirement of arrestin activity for desensitization is revealed more clearly. Overall, loss of the ability to properly modulate sensory reception can have deleterious effects on important processes, such as discrimination and habituation, which are crucial to an organism's interaction with its environment (Byk et al., 1993; Dolph et al., 1993; Kelliher et al., 2003).

It is possible this variability in arrestin mutant phenotypes may reflect the repertoire of arrestin family members and redundancy in olfactory signaling. Whereas *arr1* and *arr2* are classified as sensory arrestins, two other arrestins exist in *D. melanogaster*. A "non-visual" arrestin, *kurtz*, was identified as a lethal developmental mutation in the central nervous system and is required in several pathways (Roman et al., 2000). Furthermore, *kurtz* is expressed in antennae and could be involved in desensitization of particular odorant receptors (Roman et al., 2000; Merrill et al., 2002, 2003). An additional arrestin gene, CG32683, has recently been identified in the *Drosophila* genome project. Whereas completely uncharacterized in *Drosophila*, its ortholog in *An. gambiae* (*AgArr4*) is expressed in olfactory tissues (Merrill et al., 2003), raising the possibility that CG32683 could also be in *Drosophila* antennae. It is feasible that the presence of multiple arrestins is one reason none of the mutations completely disrupted olfactory signaling in amplitude analysis, and why kinetic and adaptive phenotypes were either absent or mild.

In order to more fully determine the extent of arrestin function in the fly olfactory system, a larval chemotaxis study was employed to measure olfactory behavioral output in arrestin mutants. Whereas earlier studies using Northern blots failed to detect *arr1* expression in prepupal stages (Smith et al., 1990), significantly more sensitive RT-PCR based mRNA analyses were able to detect both *arr1* and *arr2* expression throughout larval development in *D. melanogaster* (data not shown). Because the larval chemotaxis assay involves a locomotor-based response, flies with the $arr2^5$ allele (including double mutants) were precluded from this assay due to their seemingly decreased locomotor capacity (data not shown).

In most cases, impaired *arr1* function results in an inability to properly perceive the odor stimulus. The pattern of behavioral phenotypes is consistent with that of the adult physiological assays in terms of which odorant pathways are more severely affected. Whereas we cannot eliminate the possibility that arrestins are active in some aspect of central nervous system processing of the odorant cue, it is likely that at least some degree of disruption occurs in the periphery based on our physiological data. Whereas the olfactory system, and indeed, the nervous system as a whole, undergoes significant reconstruction between larval stages and adulthood, the overall organization is highly similar between the two life stages (Stocker, 1994). It is apparent from these studies that sensory arrestins are required in both larval and adult olfactory systems.

The discovery that arrestin mutations cause extensive impairments of olfactory behavior is especially intriguing in light of recent results showing that while the loss of a single odorant receptor leads to dramatic defects in peripheral physiology, the overall behavior of the mutant remains unaffected (Elmore et al., 2003). The widespread behavioral deficits observed in arrestin mutants indicate that they may form an important "bottleneck" in olfactory signaling.

In order to rule out any role of differential genetic backgrounds or other strain effects as a potential underlying basis for the olfactory defects, we carried out phenotypic rescue experiments with wild-type *arr1* transgenes. In these studies, pan-neuronal expression of *arr1* fully rescues both the physiological and behavioral defects of arrestin mutant responses. The ability of wild type *arr1* transgenes to restore olfactory responses provides compelling evidence that the loss-of-function arrestin alleles are the causal elements for the olfactory phenotypes reported here.

Whereas these results clearly indicate that sensory arrestins are needed for olfactory performance, the actual mechanism underlying the role of arrestins in these systems is not immediately apparent. Arrestins act in at least three capacities: desensitization, internalization, and signaling. (Pierce and Lefkowitz, 2001). Whereas it is possible that deficits in any of these would impair olfactory signaling, the most likely rationale is arrestins' role in GPCR desensitization (Krupnick and Benovic, 1998). Evidence for this model is derived from vertebrate studies of cultured olfactory neurons in which the addition of peptides that acutely disrupt arrestin-receptor interactions causes loss of desensitization upon odorant stimulation (Dawson et al., 1993; Boekhoff et al., 1994). Alternatively, it is possible that disruption of receptor recycling or MAPK signaling accounts for the faulty olfactory signaling observed here. Indeed, impairing

MAPK function alters olfactory behavior in *C. elegans* (Hirotsu et al., 2003). However, as each of these rationales requires interactions between several signal transduction components, it is difficult to segregate which aspect may be of primary importance in olfactory signaling. Future experiments are aimed at resolving these possibilities.

The olfactory deficits exhibited by arrestin mutant *Drosophila* may be counterintuitive upon first inspection. If there is loss of function of a protein that turns pathways off, one might imagine mutants would appear "more on"; that is, they may be expected to have increased olfactory responsiveness. Such a pattern is observed when arrestin function is acutely eliminated with peptide reagents (Dawson et al., 1993; Boekhoff et al., 1994). Nevertheless, activity-dependent changes in the olfactory system as a consequence of arrestin mutations can account for the observed phenotypes. If loss of arrestin function negatively impacts odorant receptor desensitization, this might result in a high degree of "leaky" background activation. As such, receptor stimulation would yield a reduced signal-to-noise ratio corresponding to the lowered physiological and behavioral responses reported here. However, if membrane excitability is so affected, stimulus-induced cellular hyperactivity may lead to neuronal degeneration, as is seen in the visual system of arrestin mutant *Drosophila* (Dolph et al., 1993); as no gross pathological changes are seen in the olfactory system of arrestin mutants (Merrill et al., 2002), significant hyper-excitability is probably not occurring.

A second possibility is that chronic loss of arrestin function leads to developmental compensation through heterologous down-regulation of the olfactory system. Indeed, in mouse knock-outs of the chemosensory-specific GRK3, decreased olfactory responsiveness results from down-regulation of signaling proteins in reparation for loss of odorant receptor desensitization (Peppel et al., 1997). This hypothesis is consistent with the physiological and behavioral phenotypes observed in arrestin-deficient *Drosophila.*

This report supports the hypothesis that sensory arrestin activity is crucial for proper olfactory performance in *D. melanogaster* and presumably other insects. Because peripheral olfactory physiology and overall behavioral responsiveness are disrupted in an odorant-specific manner in arrestin mutant flies, it is apparent that there are differential requirements for arrestin activity depending upon the signaling cascade activated by a particular odorant. The spectrum of arrestin requirements presents an additional level of complexity in peripheral olfactory coding of distinct chemical stimuli. These features make arrestins a particularly enticing target for disrupting critical chemosensory-based behaviors of insects that act as agricultural pests and vectors for human and animal diseases such as malaria.

We thank Drs. C. Desai, P. Dolph, P. Kołodziej, and M. Ramaswami for fly stocks. We also thank Drs. Kendal Broadie, A. Nicole Fox, Terry Page, and members of the Zwiebel lab for critical reading of this manuscript, as well as Ms. Lucy Hight for assistance with behavioral assays and fly maintenance.

REFERENCES

Alcorta E. 1991. Characterization of the electroantennogram in Drosophila melanogaster and its use for identifying olfactory capture and transduction mutants. J Neurophysiol 65:702–714.

Alloway PG, Dolph PJ. 1999. A role for the light-dependent phosphorylation of visual arrestin. Proc Natl Acad Sci USA 96:6072–6077.

Araneda RC, Kini AD, Firestein S. 2000. The molecular receptive range of an odorant receptor. Nat Neurosci 3:1248–1255.

Boekhoff I, Inglese J, Schleicher S, Koch WJ, Lefkowitz RJ, Breer H. 1994. Olfactory desensitization requires membrane targeting of receptor kinase mediated by beta gamma-subunits of heterotrimeric G proteins. J Biol Chem 269:37–40.

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.

Byk T, Bar-Yaacov M, Doza YN, Minke B, Selinger Z. 1993. Regulatory arrestin cycle secures the fidelity and maintenance of the fly photoreceptor cell. Proc Natl Acad Sci USA 90:1907–1911.

Campos AR, Rosen DR, Robinow SN, White K. 1987. Molecular analysis of the locus elav in Drosophila melanogaster: a gene whose embryonic expression is neural specific. EMBO J 6:425–431.

Dawson TM, Arriza JL, Jaworsky DE, Borisy FF, Attramadal H, Lefkowitz RJ, Ronnett GV. 1993. Beta-adrenergic receptor kinase-2 and beta-arrestin-2 as mediators of odorant-induced desensitization. Science 259:825–829.

Dolph PJ, Ranganathan R, Colley NJ, Hardy RW, Socolich M, Zuker CS. 1993. Arrestin function in inactivation of G protein-coupled receptor rhodopsin in vivo. Science 260: 1910–1916.

Elmore T, Ignell R, Carlson JR, Smith DP. 2003. Targeted mutation of a Drosophila odor receptor defines receptor requirement in a novel class of sensillum. J Neurosci 23:9906–9912.

Firestein S. 2001. How the olfactory system makes sense of scents. Nature 413:211–218.

Fox AN, Pitts RJ, Zwiebel LJ. 2002. A cluster of candidate

odorant receptors from the malaria vector mosquito, Anopheles gambiae. Chem Senses 27:453–459.

Ge L, Ly Y, Hollenberg M, DeFea K. 2003. A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. J Biol Chem 278:34412–34426.

Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL. 1996. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. Nature 383:447–450.

Heimbeck G, Bugnon V, Gendre N, Haberlin C, Stocker RF. 1999. Smell and taste perception in Drosophila melanogaster larva: toxin expression studies in chemosensory neurons. J Neurosci 19:6599–6609.

Hill CA, Fox AN, Pitts RJ, Kent LB, Tan PL, Chrystal MA, Cravchik A, Collins FH, Robertson HM, Zwiebel LJ. 2002. G protein-coupled receptors in Anopheles gambiae. Science 298:176–178.

Hirotsu T, Saeki S, Yamamoto M, Iino Y. 2003. The Ras-MAPK pathway is important for olfaction in Caenorhabditis elegans. Nature 404:289–293.

Kelliher KR, Ziesmann J, Munger SD, Reed RR, Zufall F. 2003. Importance of the CNGA4 channel gene for odor discrimination and adaptation in behaving mice. Proc Natl Acad Sci USA 100:4299–4304.

Krupnick JG, Benovic JL. 1998. The role of receptor kinases and arrestins in G protein-coupled receptor regulation [in process citation]. Annu Rev Pharmacol Toxicol 38:289–319.

Krupnick JG, Gurevich VV, Benovic JL. 1997. Mechanism of quenching of phototransduction. Binding competition between arrestin and transducin for phosphorhodopsin. J Biol Chem 272:18125–18131.

Laporte SA, Oakley RH, Holt JA, Barak LS, Caron MG. 2000. The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. J Biol Chem 275:23120–23126.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. 1999. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes [see comments]. Science 283: 655–661.

Luttrell LM, Lefkowitz RJ. 2002. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 115:455–465.

Merrill CE, Pitts RJ, Zwiebel LJ. 2003. Molecular characterization of arrestin family members in the malaria vector mosquito, Anopheles gambiae. Insect Mol Biol 12: 641–650.

Merrill CE, Riesgo-Escovar J, Pitts RJ, Kafatos FC, Carlson JR, Zwiebel LJ. 2002. Visual arrestins in olfactory pathways of Drosophila and the malaria vector mosquito Anopheles gambiae. Proc Natl Acad Sci USA 99:1633–1638.

Peppel K, Boekhoff I, McDonald P, Breer H, Caron MG, Lefkowitz RJ. 1997. G protein-coupled receptor kinase 3 (GRK3) gene disruption leads to loss of odorant receptor desensitization. J Biol Chem 272:25425–25428.

Pierce KL, Lefkowitz RJ. 2001. Classical and new roles of β-arrestins in the regulation of G-protein-coupled receptors. Nat Rev Neurosci 2:727–733.

Pierce KL, Luttrell LM, Lefkowitz RJ. 2001. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene 20:1532–1539.

Rakhit S, Pyne S, Pyne NJ. 2001. Nerve growth factor stimulation of p42/p44 mitogen-activated protein kinase in PC12 cells: role of G(i/o), G protein-coupled receptor kinase 2, beta-arrestin I, and endocytic processing. Mol Pharmacol 60:63–70.

Raming K, Freitag J, Krieger J, Breer H. 1993. Arrestin-subtypes in insect antennae. Cell Signal 5:69–80.

Riesgo-Escovar JR, Woodard C, Carlson JR. 1994. Olfactory physiology in the Drosophila maxillary palp requires the visual system gene rdgB. J Comp Physiol [A] 175: 687–693.

Roman G, He J, Davis RL. 2000. Kurtz, a novel nonvisual arrestin, is an essential neural gene in Drosophila. Genetics 155:1281–1295.

Rubin GM, Spradling AC. 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science 218:348–353.

Smith DP, Shieh BH, Zuker CS. 1990. Isolation and structure of an arrestin gene from Drosophila. Proc Natl Acad Sci USA 87:1003–1007.

Stocker RF. 1994. The organization of the chemosensory system in *Drosophila melanogaster* : a review. Cell Tissue Res 275:3–26.

Stortkuhl KF, Hovemann BT, Carlson JR. 1999. Olfactory adaptation depends on the Trp Ca2+ channel in Drosophila. J Neurosci 19:4839–4846.

Tan M, Groszer M, Tan MA, Pandya A, Liu X, Xie CW. 2003. Phosphoinositide 3-kinase cascade facilitates mu-opiod desensitization in sensory neurons by altering G protein-effector interactions. J Neurosci 23:10292–10301.

Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM. 2002. Beta-arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensis AT1a receptor stimulation. J Biol Chem 277:9429–9436.

Wistow G, Katial A, Craft C, Shinohara T. 1986. Sequence analysis of bovine retina S-antigen. Relationships with alpha-transducin and G-proteins. FEBS Lett 196:23–28.

EMBO *reports*

scientific report

Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the *Defensin* gene

Stéphanie Blandin, Luis F. Moita, Thomas Köcher, Matthias Wilm, Fotis C. Kafatos & Elena A. Levashina+

European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, D-69117 Heidelberg, Germany

Received May 24, 2002; revised July 18, 2002; accepted July 19, 2002

Anopheles gambiae, the major vector of human malaria parasite, is an important insect model to study vector–parasite interactions. Here, we developed a simple _in vivo_ double-stranded RNA (dsRNA) knockout approach to determine the function of the mosquito antimicrobial peptide gene _Defensin_. We injected dsRNA into adults and observed efficient and reproducible silencing of _Defensin_. Analysis of the knockdown phenotype revealed that this peptide is required for the mosquito antimicrobial defense against Gram-positive bacteria. In contrast, in mosquitoes infected by _Plasmodium berghei_, no loss of mosquito viability and no significant effect on the development and morphology of the parasite midgut stages were observed in the absence of Defensin. We conclude that this peptide is not a major antiparasitic factor in _A. gambiae in vivo_. Our results open new perspectives for the study of mosquito gene function _in vivo_ and provide a basis for genome-scale systematic functional screens by targeted gene silencing.

INTRODUCTION

Anopheles gambiae is the most important vector for *Plasmodium falciparum* malaria in sub-tropical Africa and thus a critical link in the transmission cycle of one of the most serious infectious diseases of humanity (Greenwood and Mutabingwa, 2002). In recent years, this mosquito has been studied intensively by the methods of molecular and cell biology, with a special emphasis on innate immune mechanisms possibly implicated in limiting the load of parasite transmission. Such studies promise to advance rapidly when the completion of the *A. gambiae* genome sequencing reveals the universe of genes upon which mosquito immunity resides (Hoffman *et al.*, 2002). Despite the existence of a good microsatellite-based genetic map (Zheng *et al.*, 1996) and robust techniques for germ-line transgenesis (Catteruccia *et al.*, 2000; Grossman *et al.*, 2001), inherent limitations of mosquito stock maintenance hinder the traditional methods for gene function analysis, such as large mutagenesis screens, fine-scale gene mapping and transgenic analysis. The double-stranded RNA (dsRNA) interference is potentially adaptable to systematic reverse genetic screens (Gonczy *et al.*, 2000), and we have shown recently that it can be used to assess gene function in cultured mosquito cells (Levashina *et al.*, 2001). Here, we demonstrate that dsRNA can also be used to disrupt essential gene function in the whole mosquito.

When dsRNA is taken up by cells, it is cleaved into small interfering fragments that can trigger specific degradation of the endogenous target mRNA (Zamore *et al.*, 2000). *Caenorhabditis elegans* is the only multicellular organism where the direct injection of dsRNA in the animal has been demonstrated to result in gene silencing throughout development (Fire *et al.*, 1998). In *Drosophila*, dsRNA injection in embryos is widely used for assessing gene function in early development, but in adult flies RNA interference is routinely mediated by the expression of hairpin dsRNA in transgenic strains (St Johnston, 2002), a technique that would be very difficult to apply systematically in mosquitoes. Instead, we have opted for a rapid and direct approach, intrathoracic injection of dsRNA in adult *A. gambiae*, to bring about efficient and reproducible silencing of the gene encoding the antimicrobial peptide, Defensin, which is encoded by a single gene in the *A. gambiae* genome.

So far, three antimicrobial peptides have been characterized in *A. gambiae*, Defensin, Cecropin and Gambicin, which are produced by the fat body and hemocytes and secreted into hemolymph upon immune challenge (Richman *et al.*, 1996; Vizioli *et al.*, 2000, 2001a). These polypeptides exhibit bactericidal and/or fungicidal activities *in vitro* and are thought to constitute the first line of defense against microbial infections

+Corresponding author. Tel: +49 6221 387 440; Fax: +49 6221 387 211; E-mail: elena.levashina@embl-heidelberg.de

(reviewed in Dimopoulos *et al.*, 2001; Hoffmann and Reichhart, 2002). Related peptide families exist in vertebrates and are mostly expressed by epithelia and leukocytes, acting locally to limit bacterial infection (reviewed in Lehrer and Ganz, 2002). A knockout mouse lacking the Defensin-like peptide Cathelicidin has been shown recently to be susceptible to necrotic skin infections caused by Gram-positive Group A streptococci (Nizet *et al.*, 2001). In *Drosophila*, the inactivation of two major regulatory signalling pathways, *Toll* and *Imd*, has been used to turn off large groups of immune genes, including the anti-microbial peptide genes, and to associate them collectively with *in vivo* antifungal and antibacterial functions, respectively (Lemaitre *et al.*, 1996). However, no loss-of-function mutants for individual antimicrobial peptide genes have been reported as yet.

The expression of *Defensin* is predominantly induced in the mosquito fat body shortly after bacterial challenge. It is also induced locally in the midgut and salivary gland epithelia upon invasion by malaria parasites, suggesting that Defensin may have a broad role in the defense against both microbes and parasites (Richman *et al.*, 1996, 1997). This presumption was supported by *in vitro* tests of antiparasitic activity and by injection studies in *Aedes* mosquitoes infected by avian malaria (Shahabuddin *et al.*, 1998). However, rigorous conclusions about Defensin function *in vivo* require analysis by a loss-of-function approach in the intact mosquito.

RESULTS AND DISCUSSION

To knock down the *Defensin* gene expression, we injected 1- to 2-day-old females with dsRNA corresponding to the genes for either Defensin (*dsDEF*) or green fluorescent protein (*dsGFP*) as a control and allowed the mosquitoes to recover for 4 days. The dsRNA-treated mosquitoes were then challenged with *Escherichia coli*, and the presence/absence of the *Defensin* transcripts was monitored from day 1 to 8 by RNA blotting and RT–PCR (Figure 1). In six independent experiments, the injection of either Gram-negative *E. coli* or Gram-positive *Staphylococcus aureus* induced the expression of *Defensin* mRNA in non-treated control mosquitoes (Figure 1A; data not shown). We observed that the injection of *dsGFP* partially suppressed the ultimate level of *Defensin* induction after bacterial challenge (Figure 1A versus C), and therefore we used *dsGFP* mosquitoes as controls throughout this study. In the *dsDEF* mosquitoes, no *Defensin* mRNA of proper size was detected already at day 5 after dsRNA injection (Figure 1B and C, day 1 after bacterial challenge). Instead, a strong faster migrating signal was consistently present, accompanied by a faint signal migrating more slowly than *Defensin* mRNA. We interpret these signals as denatured and non-denatured dsRNA, respectively, as they are also exhibited by the input dsRNA (asterisks in Figure 1D). Signals corresponding to the input *dsGFP* were also detected using the *GFP* probe (bottom asterisk in Figure 1C and D), indicating that dsRNAs are stable for at least 12 days after injection in mosquitoes and therefore can provide a long-lasting inhibition of endogenous gene expression. In contrast to robust induction of *Defensin* in *dsGFP* mosquitoes (Figure 1E, days 1, 2 and 4), only traces of *Defensin* mRNA were detected in *dsDEF* mosquitoes using sensitive RT–PCR and primers corresponding to the 5′ and 3′ UTRs of the *Defensin* gene. We conclude that the injection of specific dsRNA successfully inhibits induction of *Defensin* after bacterial challenge.

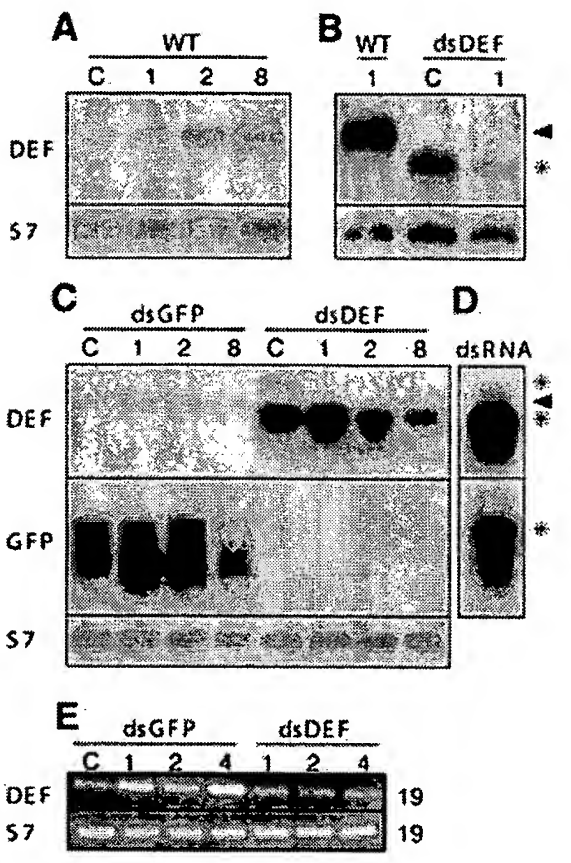


Fig. 1. RNA analysis of the *Defensin* gene knockout by dsRNA. RNA blots demonstrate that, over a period from 1 to 8 days (numbers above the images), *E. coli* challenge stably induces the expression of *Defensin* (*DEF*) mRNA (arrowheads) above the uninfected control level (C) in wild-type (WT) (**A** and **B**) and in *dsGFP*-treated, but not in *dsDEF*-treated, mosquitoes (B and **C**). In (**D**), the input *dsRNAs* match in size the signals (*DEF* and *GFP*) that are detected in dsRNA-injected mosquitoes (asterisks). (A), (C) and (D) were run on 1.2% agarose gels and (B) on an 1.4% agarose gel. The ribosomal protein *S7* transcript was used as a loading control. *GFP*, green fluorescent protein. (**E**) RT–PCR analysis of the *DEF* (19 cycles) gene expression in *dsGFP*- and *dsDEF*-treated mosquitoes before (C) and after (days 1, 2 and 4) bacterial challenge. The expression of the ribosomal protein gene *S7* (19 cycles) served as control.

The efficacy of the *Defensin* knockout was further validated at the polypeptide level by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectometry (MS) of hemolymph samples (Figure 2A). Although the *dsDEF* and *dsGFP* peptide profiles were mostly comparable, the latter showed a strong peak at 4136.2 Da, which was undetectable in the *dsDEF* sample and corresponded to singly protonated *A. gambiae* Defensin bearing three disulfide bridges (calculated mass 4136.7 Da) (Vizioli *et al.*, 2001b). This identification was confirmed by sequencing of the reduced peptide (data not shown). We also followed the *Defensin* knockout in the epithelium of the anterior midgut 24 h after infectious bloodmeal. The *dsGFP* and *dsDEF* midgut extracts again differed by a prominent Defensin peak at 4136.9 Da, which was present only in the midgut cells of *dsGFP* mosquitoes (Figure 2B). Thus, the injection of dsRNA in adult mosquitoes disrupts the expression of the targeted gene at both the RNA and polypeptide levels. Our data demonstrate that dsRNA knockout is efficient in the three different

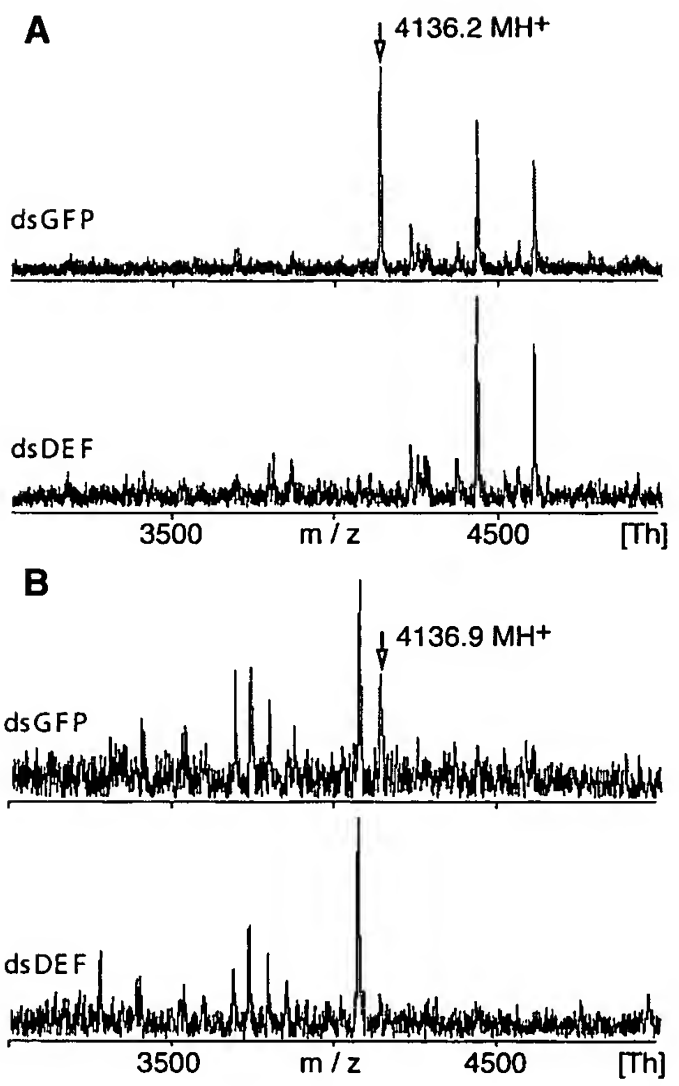


Fig. 2. MALDI-TOF MS analysis of the *Defensin* gene knockout by dsRNA. **(A)** Mosquitoes were challenged with a mixture of *E. coli* and *S. aureus*, and 36 h later the presence of Defensin peptide was detected in the hemolymph of control *dsGFP*, but not of *dsDEF*, mosquitoes. **(B)** Defensin is present in the anterior midgut 24 h after infectious bloodmeal in control *dsGFP*, but not in *dsDEF*, mosquitoes. The peaks corresponding to Defensin and their molecular weights are indicated by arrows.

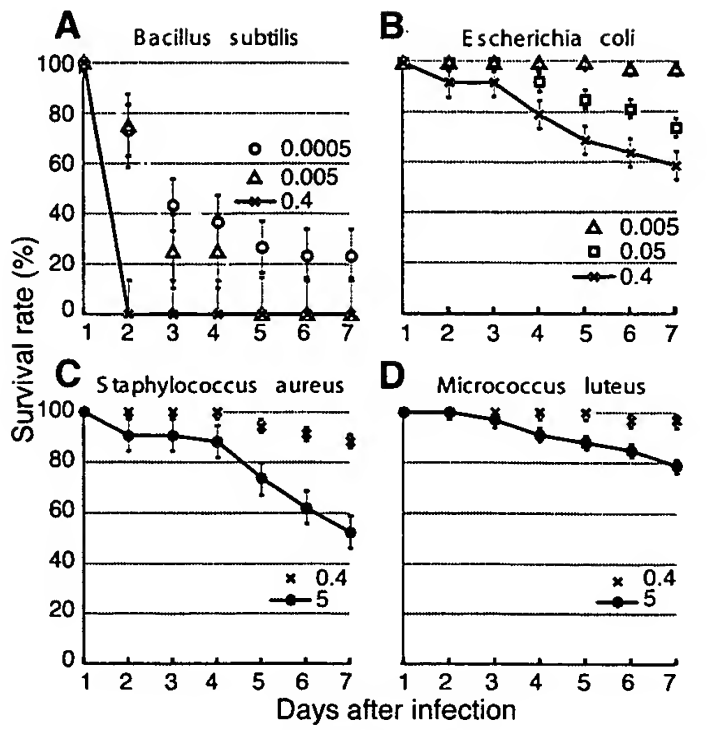


Fig. 3. Lethality of control *dsGFP*-treated mosquitoes after infection with different doses of bacteria. Survival rates (%) are presented for mosquitoes infected with **(B)** the Gram-negative bacterium *E. coli* or the Gram-positive bacteria **(A)** *B. subtilis*, **(C)** *S. aureus* and **(D)** *M. luteus*. The bacterial concentrations are expressed as optical densities (OD) of suspensions at 600 nm: OD_{600} = 0.0005 (open circles), OD_{600} = 0.005 (open triangles), OD_{600} = 0.05 (open squares), OD_{600} = 0.4 (crosses) and OD_{600} = 5 (asterisks). Each experiment was performed with 50 mosquitoes for each bacterial species, and the results shown are representative of three independent experiments. Standard errors are indicated by bars.

cell types tested (midgut, fat body and hemocytes), which originate from two distinct cell lineages: endoderm and mesoderm.

The efficacy, effectiveness and reproducibility of the knockout allowed us to determine the function and specificity of Defensin *in vivo*. Because of *in vitro* indications that Defensin is particularly potent against Gram-positive bacteria (Vizioli *et al.*, 2001b), we focused on three members of this class, *S. aureus*, *Micrococcus luteus* and *Bacillus subtilis*, as well as on a Gram-negative species, *E. coli*. We injected measured bacterial suspensions of each species into control *dsGFP A. gambiae* females and followed the survival of the mosquitoes for 7 days (Figure 3). The bacteria exhibited different degrees of pathogenicity that were independent of Gram classification. *Bacillus subtilis* rapidly killed *A. gambiae* even at concentrations as low as 85 bacteria per mosquito (Figure 3A). The next most efficient pathogen, *E. coli*, caused comparable mosquito lethality only when injected in 10-fold higher numbers; *S. aureus* was substantially less pathogenic, and *M. luteus* was the least effective (Figure 3B–D). Differential susceptibility to bacteria has also been reported in *Drosophila melanogaster*, where, surprisingly, *S. aureus*, as well as *B. subtilis*, cause rapid death, while *M. luteus* is again only weakly pathogenic (Tzou *et al.*, 2002). Comparative analysis of bacterial pathogenicity may be fruitful in pinpointing specificities of the immune response in related dipteran species.

These experiments allowed us to define the sublethal concentrations for infection with the three bacterial species other than *B. subtilis*. We injected the selected concentrations of bacteria in both *dsDEF* and control *dsGFP* females and followed their respective survival rates over a period of 7 days (Figure 4). The profiles did not differ significantly in the case of *E. coli*- and mock-injected mosquitoes. In contrast, the *dsDEF* mosquitoes were modestly susceptible to *M. luteus* (20% lethality) and highly susceptible to *S. aureus* (80% lethality). This is the first demonstration *in vivo* that a single endogenous immune peptide, Defensin, is necessary for the resistance of a mosquito to two Gram-positive bacteria and not to a Gram-negative species of bacteria. The plateau in mortality that is seen in *dsDEF* mosquitoes 5 days after infection by *M. luteus* suggests that additional immune factors or cellular processes may be implicated in the clearance of this mild pathogen.

We next examined the potential role of Defensin in the immune response of *A. gambiae* to the rodent malaria parasite, *Plasmodium berghei*. *Defensin* is constitutively expressed in the anterior midgut epithelium and is further induced by malaria

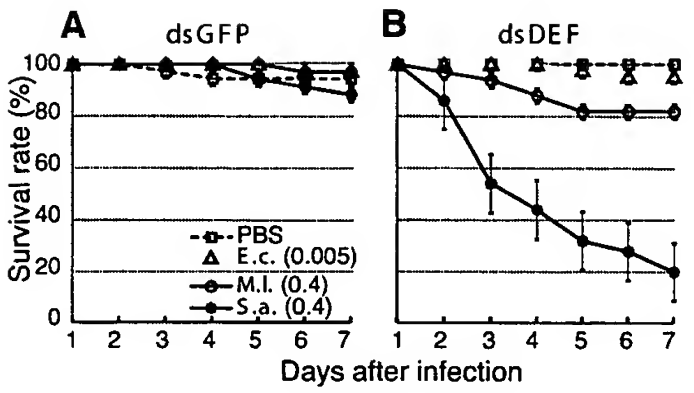


Fig. 4. *Defensin* knockdown mosquitoes are susceptible to Gram-positive, but not to Gram-negative, bacteria. The survival rates (%) of (**A**) *dsGFP*-treated mosquitoes and (**B**) *dsDEF*-treated mosquitoes after injection of PBS (open squares), *E. coli* (OD_{600} = 0.005, open triangles), *M. luteus* (OD_{600} = 0.4, open circles) or *S. aureus* (OD_{600} = 0.4, asterisks) are shown. Each experiment was performed with 50 mosquitoes for each bacterial species, and the results shown are representative of three independent experiments. Standard errors are indicated by bars.

infection (Richman *et al.*, 1997; Vizioli *et al.*, 2001b). In *Aedes aegypti*, the injection of high doses of related Defensins from a dragonfly and a fleshfly at specific time points after an infectious bloodmeal interfered with the development of the midgut stages of the parasite (Shahabuddin *et al.*, 1998). These studies prompted us to monitor parasite numbers during *P. berghei* infections in *dsGFP* and *dsDEF* mosquitoes, using as a readout the number of oocysts per midgut 10–12 days after infection. We reasoned that, if endogenous Defensin acts antiparasitically, its absence during development of the *Plasmodium* midgut stages would remove a constraint and result in higher parasite loads. We observed that the mean number of oocysts per midgut was unchanged or even slightly lower in *dsDEF* than in *dsGFP* mosquitoes (Table I). Moreover, parasite-infected *dsDEF* and *dsGFP* mosquitoes showed no significant differences in mosquito viability, ookinete/oocyst morphology or the frequency distribution of oocyst numbers. We conclude that malaria-induced endogenous *A. gambiae* Defensin does not act as a significant antiparasitic factor *in vivo*.

In conclusion, the simple and convenient dsRNA technique that we describe here efficiently disrupts gene function in distinct tissues of adult mosquitoes. As a proof of principle, we have used this method to delimit phenotypically the *in vivo* function of a single immune peptide against different types of infections, showing it to play an important role in the resistance to Gram-positive bacteria. Evidently, antimicrobial peptides that would be unaffected by *dsDEF* injection cannot substitute for Defensin. Recently, we have successfully applied dsRNA to knock out 20 additional immune genes in *A. gambiae* (data not shown), thus confirming the general validity of this method in the study of immune responses in the mosquito. With a reverse genetics method now in hand, it should be possible to conduct systematic functional genomic analysis in this major vector of human malaria.

METHODS

Mosquito colony. *Anopheles gambiae* strain G3 was reared as described previously (Richman *et al.*, 1996).

Double-stranded RNA preparation and injection in mosquitoes. dsRNAs were produced as described previously using the plasmids pLL6ds for control *dsGFP* (Levashina *et al.*, 2001) and pLL80 for *dsDEF*. pLL80 was constructed in two steps. *Defensin* cDNA of 404 bp was PCR-amplified using *dfn a* and *dfn b* primers (Richman *et al.*, 1996) and cloned into pCR2.1-TOPO (Invitrogen), resulting in pLL79. The 515 bp *Hin*dIII–*Xba*I fragment of pLL79 was then subcloned between the two T7 promoters of pLL10. Sense and antisense RNAs were synthesized using the T7 Ambion kit, annealed in water and stored as dsRNAs at –80°C until use. A nano-injector (Nanoject, Drummond) was used to introduce 69 nl of dsRNAs (1 mg/ml) in water in the thorax of CO_2-anesthetized mosquito females, which were then allowed to recover for 4 days.

RNA analysis. Total RNA was extracted from 15 mosquitoes with TRIzol Reagent (Invitrogen) and separated by electrophoresis. Two different conditions were used: to ascertain the absence of endogenous *Defensin* transcripts, the electrophoresis were performed for 6 h using 1.2% agarose gels (Figure 1B); clear signals corresponding to input dsRNAs were detected using 1.4% agarose gels and 4 h migration time (Figure 1A, C and D). Separated total RNAs were transferred to a nylon membrane (Hybond-N$^+$, Amersham Pharmacia Biotech). Blots were hybridized sequentially with the radioactively labeled probes [Ready-To-Go DNA labeling beads (dCTP), Amersham Pharmacia Biotech]: *Defensin* (pLL79 insert), *S7* (Salazar *et al.*, 1993) and *GFP* (pLL6 insert).

For RT–PCR analysis, *Defensin*-specific primers were selected that do not overlap with the dsRNA used for the knockout: 5′-UTR primer, 5′-AAC TCC AGC CAA GCT AAA GC-3′; and 3′-UTR primer, 5′-GAA TTA AGC CTG TGT TGT AAA C-3′. Total RNA was extracted as above from whole mosquitoes at the

Table I. Survival of *P. berghei* oocysts in the *dsGFP*- and *dsDEF*-treated mosquitoes

Experiment	dsRNA	Number of midguts	Oocyst number per midgut			Mean ± SE[a] (oocysts per midgut)
			0	1–29	>30	
1	*dsGFP*	19	5	9	5	16.74 ± 3.7
	dsDEF	24	7	14	3	10.75 ± 3.1
2	*dsGFP*	13	4	9	0	2.77 ± 0.6
	dsDEF	25	7	18	0	2.48 ± 0.4

[a]SE, standard error.

indicated time points. S7-specific primers and the RT–PCR conditions were as described previously (Richman *et al.*, 1996). For amplification of both *S7* and *Defensin*, 19 PCR cycles were used.

Mass spectrometry. The hemolymph of 40 mosquitoes was collected by centrifugation of decapitated females (3000 r.p.m., 5 min) and used in 1/10 dilution in acidified water. Five dissected anterior midguts were homogenized in 0.5% trifluoracetic acid, 50% acetonitrile; and extracts were cleared by centrifugation. The samples were analyzed without further purification in a modified thin-layer preparation (Vorm *et al.*, 1994). MALDI-TOF MS analysis was performed on a Bruker Biflex (Bremen, Germany) mass spectrometer in linear positive mode using delayed extraction. Tandem MS with a nano-electrospray source mounted on a Micromass QTOF1 (Manchester, UK) mass spectrometer was used for peptide sequencing.

Bacterial challenge and mosquito survival. GFP-expressing *E. coli* OP-50 was a gift from J.J. Ewbank (INSERM, Marseille-Luminy, France). *Bacillus subtilis, M. luteus* and *S. aureus* were kind gifts from P. Bulet (IBMC, Strasbourg, France). Bacteria were cultured to OD_{600} = 0.4, pelleted, washed and resuspended in phosphate-buffered saline (PBS) to indicated concentrations. The number of bacteria injected was estimated by the plating of appropriate aliquots of bacterial suspension on LB plates. Mosquitoes were anesthetized with CO_2, injected into the thorax with 69 nl of the bacterial suspension or PBS for controls and allowed to recover. Mosquitoes that died within 24 h of injection were not considered in the analysis. Dead mosquitoes were daily counted and removed over a period of 7 days. The results shown here are representative of at least three independent experiments, each carried out with 50 mosquitoes per tested group.

Parasite infections and oocyst counting. Parasite infections were performed essentially as described previously (Richman *et al.*, 1997). Briefly, *P. berghei* parasites were passaged in CD1 mice, and parasitemia was determined from Giemsa-stained blood films. For each experiment, *dsGFP* and *dsDEF* mosquitoes were fed on the same infected mouse. Mosquito midguts were dissected 10–12 days later, fixed and DAPI stained. Morphology was examined, and the numbers of oocysts were counted using a UV-light fluorescent microscope (Zeiss).

ACKNOWLEDGEMENTS

We thank D. Doherty for help with the mosquito colony and J.J. Ewbank and P. Bulet for bacterial strains. This work was supported by EMBL, the National Institutes of Health and the European Commission.

REFERENCES

Catteruccia, F., Nolan, T., Loukeris, T.G., Blass, C., Savakis, C., Kafatos, F.C. and Crisanti, A. (2000) Stable germline transformation of the malaria mosquito *Anopheles stephensi*. *Nature*, **405**, 959–962.

Dimopoulos, G., Müller, H.M., Levashina, E.A. and Kafatos, F.C. (2001) Innate immune defense against malaria infection in the mosquito. *Curr. Opin. Immunol.*, **13**, 79–88.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806–811.

Gonczy, P. *et al.* (2000) Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature*, **408**, 331–336.

Greenwood, B. and Mutabingwa, T. (2002) Malaria in 2002. *Nature*, **415**, 670–672.

Grossman, G.L., Rafferty, C.S., Clayton, J.R., Stevens, T.K., Mukabayire, O. and Benedict, M.Q. (2001) Germline transformation of the malaria vector, *Anopheles gambiae*, with the piggyBac transposable element. *Insect Mol. Biol.*, **10**, 597–604.

Hoffman, S.L., Subramanian, G.M., Collins, F.H. and Venter, J.C. (2002) *Plasmodium*, human and *Anopheles* genomics and malaria. *Nature*, **415**, 702–709.

Hoffmann, J.A. and Reichhart, J.M. (2002) *Drosophila* innate immunity: an evolutionary perspective. *Nat. Immunol.*, **3**, 121–126.

Lehrer, R.I. and Ganz, T. (2002) Defensins of vertebrate animals. *Curr. Opin. Immunol.*, **14**, 96–102.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. and Hoffmann, J.A. (1996) The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell*, **86**, 973–983.

Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M. and Kafatos, F.C. (2001) Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell*, **104**, 709–718.

Nizet, V. *et al.* (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature*, **414**, 454–457.

Richman, A.M., Bulet, P., Hetru, C., Barillas-Mury, C., Hoffmann, J.A. and Kafalos, F.C. (1996) Inducible immune factors of the vector mosquito *Anopheles gambiae*: biochemical purification of a defensin antibacterial peptide and molecular cloning of preprodefensin cDNA. *Insect Mol. Biol.*, **5**, 203–210.

Richman, A.M., Dimopoulos, G., Seeley, D. and Kafatos, F.C. (1997) *Plasmodium* activates the innate immune response of *Anopheles gambiae* mosquitoes. *EMBO J.*, **16**, 6114–6119.

Salazar, C.E., Mills-Hamm, D., Kumar, V. and Collins, F.H. (1993) Sequence of a cDNA from the mosquito *Anopheles gambiae* encoding a homologue of human ribosomal protein S7. *Nucleic Acids Res.*, **21**, 4147.

Shahabuddin, M., Fields, I., Bulet, P., Hoffmann, J.A. and Miller, L.H. (1998) *Plasmodium gallinaceum*: differential killing of some mosquito stages of the parasite by insect defensin. *Exp. Parasitol.*, **89**, 103–112.

St Johnston, R. (2002) The art and design of genetic screens: *Drosophila melanogaster*. *Nat. Rev. Genet.*, **3**, 176–188.

Tzou, P., Reichhart, J.M. and Lemaitre, B. (2002) Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient *Drosophila* mutants. *Proc. Natl Acad. Sci. USA*, **99**, 2152–2157.

Vizioli, J. *et al.* (2000) Cloning and analysis of a cecropin gene from the malaria vector mosquito, *Anopheles gambiae*. *Insect Mol. Biol.*, **9**, 75–84.

Vizioli, J., Bulet, P., Hoffmann, J.A., Kafatos, F.C., Muller, H.M. and Dimopoulos, G. (2001a) Gambicin: a novel immune responsive antimicrobial peptide from the malaria vector *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA*, **98**, 12630–12635.

Vizioli, J., Richman, A.M., Uttenweiler-Joseph, S., Blass, C. and Bulet, P. (2001b) The defensin peptide of the malaria vector mosquito *Anopheles gambiae*: antimicrobial activities and expression in adult mosquitoes. *Insect Biochem. Mol. Biol.*, **31**, 241–248.

Vorm, O., Roepstorff, P. and Mann, M. (1994) Improved resolution and very high sensitivity in MALDI TOF of matrix surfaces made by fast evaporation. *Anal. Chem.*, **66**, 3281–3287.

Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, **101**, 25–33.

Zheng, L., Benedict, M.Q., Cornel, A.J., Collins, F.H. and Kafatos, F.C. (1996) An integrated genetic map of the African human malaria vector mosquito, *Anopheles gambiae*. *Genetics*, **143**, 941–952.

DOI: 10.1093/embo-reports/kvf180

JOURNAL OF NEUROPHYSIOLOGY
Vol. 65, No. 3, March 1991. *Printed in U.S.A.*

Characterization of the Electroantennogram in *Drosophila melanogaster* and its use for Identifying Olfactory Capture and Transduction Mutants

ESTHER ALCORTA
Max-Planck-Institut fuer biologische Kybernetik, 7400 Tuebingen, Federal Republic of Germany

SUMMARY AND CONCLUSIONS

1. Amplitude as well as time course of the electroantennogram (EAG) in *Drosophila* has been used for describing electrical changes produced in the antenna in response to odorous stimulation.

2. Maximal amplitude of response appears to be directly correlated to stimulus concentration but, after achieving a maximum value, is independent of stimulation duration.

3. Rise time and fall time constants have been quantified for describing kinetics of response. The rise time constant decreases, but the fall time constant increases when increasing concentrations of odorant are supplied.

4. Variation among individuals for these EAG parameters is small enough to uncover even partial defects affecting the first sensory step. This fact combined with the possibility of obtaining mutants with defects in any intermediate process producing the electrical response makes the EAG of *Drosophila* a very useful tool for dissecting the components of the capture and transduction processes in the olfactory sense.

5. This kind of quantitative study of the EAG has been used in a new *Drosophila* mutant, *od A*, for localizing peripheral expression of the mutation. *od A* has been isolated as a behavioral mutant with an abnormally enhanced olfactory response to ethyl acetate.

6. The mutant's EAG in response to this odorant displays a normal amplitude but abnormal kinetics. Rise time as well as fall time show slower kinetics than normal, suggesting some defective step in the capture and transduction process.

INTRODUCTION

Insects perceive scents mainly with their antennae. Odorant molecules excite olfactory neurons in the sensilla of the third antennal segment (Kaissling 1971). The axons of these primary sensory cells extend directly to the CNS (Power 1946). Electrophysiological measurements at the antennal level give functional information about the capture of odorant molecules and the transduction of the external chemical signal to a corresponding electrical signal in the olfactory neurons. They have been extensively used in insects, particularly for studying moths' response to pheromones, with whole antenna (electroantennogram, EAG) as well as nerve and single sensilla recordings (Kaissling 1987).

The small size of *Drosophila melanogaster* makes electrophysiological analysis more difficult. However, the information about olfaction obtained from other species can be supplemented in *Drosophila* by using a genetic approach. The possibility of disrupting normal sensory activity by modifying single steps, even at the molecular level, and testing their effects has made genetic mutations a powerful tool for this kind of study. Mutations disrupting capture and transduction at different levels could help to dissect the primary olfactory reaction into single components and even to correlate the effects of failures in these single components with an abnormal behavioral response.

In *Drosophila*, the genetic approach has been used extensively to dissect the visual system (see, for example, Heisenberg and Goetz 1975; Hall 1982; Hall and Greenspan 1979; Heisenberg 1979; Pak 1975). Recently, attention has been focused on the olfactory system. Besides mutations affecting morphology of the major components of olfactory reception, such as antennae (*Antennapedia* and *engrailed*) or antennal sensilla (*lozenge*), new olfactory behavioral mutants without apparent morphological modifications have been isolated (Aceves-Piña and Quinn 1979; Helfand and Carlson 1989; McKenna et al. 1989; Rodrigues and Siddiqi 1978; Siddiqi 1987; Woodard et al. 1989). All of them show a partial loss of response to odorants, either to one class of stereochemically related molecules or to several classes of chemicals. The study of this kind of mutant offers the double possibility of locating the structure responsible for the abnormal behavior and characterizing the corresponding functional changes, as well as adding the data of how the fly translates this information into a particular behavior.

Extracellular recording at the proximal funiculus (EAG) has been used to describe the electrical response of the whole *Drosophila* antenna to airborne chemicals (Borst 1984; Venard and Pichon 1981, 1984). Qualitative differences in response to various odorants and quantitative differences in amplitude of the EAG in response to different concentrations have been reported (Venard and Pichon 1981). The existence of specific or nonspecific pathways for different kinds of molecules has also been proposed by looking at amplitude data (Borst 1984), and peripheral localization of a defect has been suggested in an olfactory behavior mutant (Venard and Pichon 1984). All these studies use amplitude as the only parameter of measure; however, a more complete quantification of the signal's dynamics is lacking, and effects in the temporal pattern of EAG response may have been missed. The olfactory behavior mutants already described (Helfand and Carlson 1989; McKenna et al. 1989; Rodrigues and Siddiqi 1978; Siddiqi 1987; Woodard et al. 1989) show a concentration-dependent phenotype and not complete anosmia. Because of this,

quantitative study of the effects of increasing concentrations of odorant in the whole time course of the EAG may also be necessary for localizing mutations at the capture and transduction level.

The present study characterizes the time course of the EAG in *Drosophila* in response to an odorant, ethyl acetate, which elicits a strong behavioral response in adults as well as in larvae. Effects of various concentrations and stimulation durations are considered, taking into account that, although both factors are related to the number of molecules that stimulate olfactory neurons, the temporal processes involved in the whole response, from the adsorption to the decomposition of the stimulant molecules, could be affected differently (Kaissling 1987).

The olfactory behavioral phenotype of the new autosomal mutation *od A* and its possible effect on antennal reception as measured by EAGs are described. Analysis of the amplitude and kinetics of the *od A* mutant's EAG has been carried out in response to different concentrations and durations of stimulation of ethyl acetate.

METHODS

EAG measurements

Canton-S females 2–5 days old were fixed to a stand and immobilized with wax. The antennogram was recorded (Fig. 1) on the proximal surface of the funiculus (Borst 1984), at the region of the antennal nerve output, with the use of a glass electrode with a tip diameter of ~10 μm filled with 0.5 M KCl solution. A tungsten wire inserted into the head capsule was used as the indifferent electrode. The electrical signal was amplified and recorded, displayed and stored by computer as voltage data with a sampling rate of 50 Hz, except where otherwise indicated. The same computer was used to control stimulus delivery.

STIMULUS DELIVERY. Olfactory stimulation was delivered with the use of a constant air flow of 3 ml/s through a 1-mm-diam tube that terminated at 0.5 cm from the antennae. An electrically triggered valve (Lee, LFAA 1200118 H) system allowed the replacement of charcoal-filtered air by the odorant stream for controlled stimulus durations. A system of five wash bottles containing either 5 ml of pure paraffin oil (control bottle) or 5 ml of different dilutions of ethyl acetate in paraffin oil (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}) was used (Fig. 1) to rapidly test the response of each fly to different concentrations.

The tested stimulus durations were 0.5, 2, and 10 s. The highest concentration (10^{-1}) was also tested for 30 s of stimulation.

A total of 10 flies were measured in the cases of 0.5 and 2 s of stimulation. Ten responses for each concentration and fly were taken, beginning with 10 repetitions of the smallest concentration and so on. The interval between repetitions was 20 and 30 s, respectively, for the two different durations of stimulation. When longer stimulation was provided (10 or 30 s of stimulation), flies were submitted only once to the different concentrations to avoid possible adaptation effects. In this case, 20 flies were tested for each concentration. Voltage sampling rate was 20 Hz for these two conditions.

MEASURED PARAMETERS. Voltage data of the whole time course of the response were corrected for baseline drift, which was

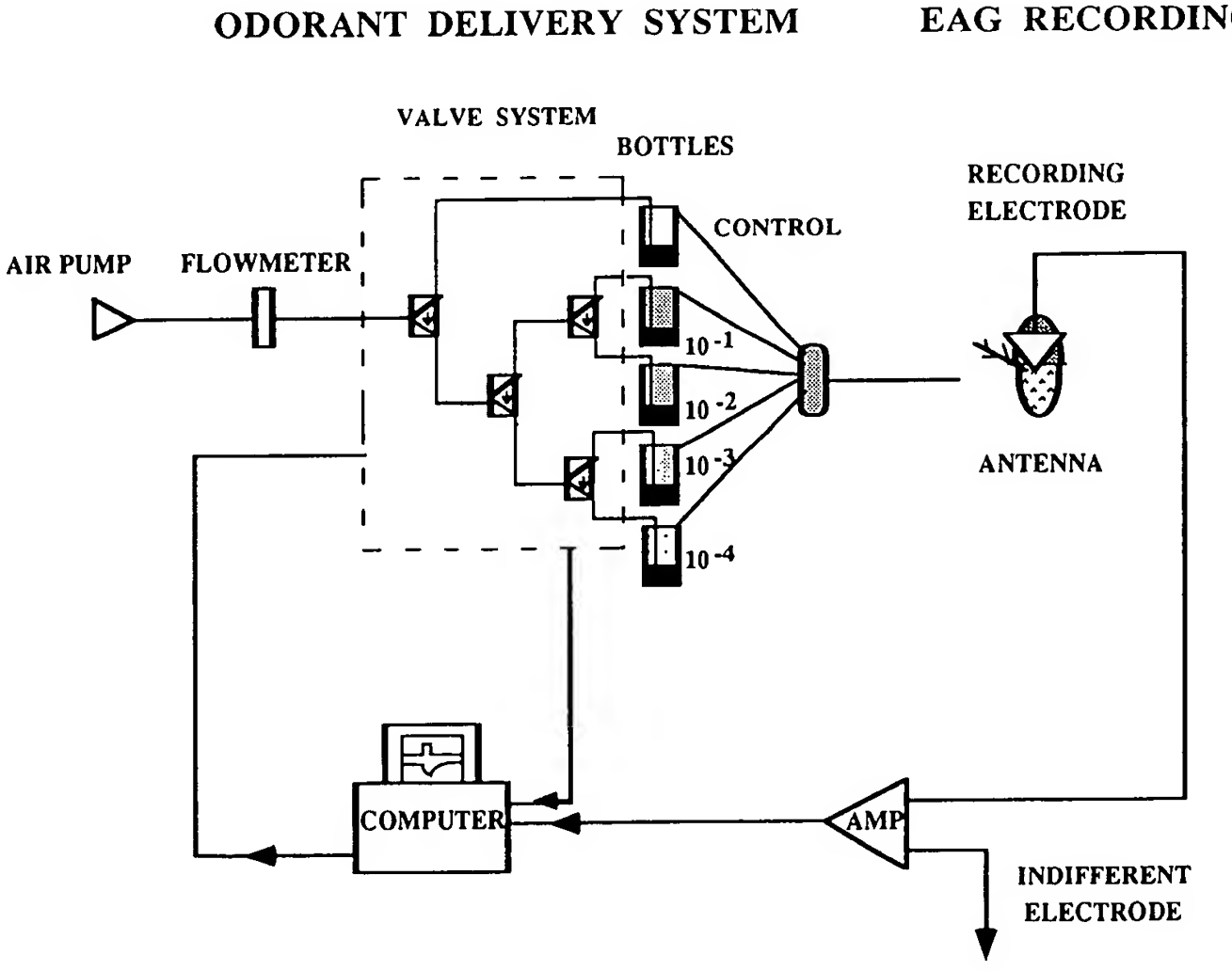


FIG. 1. Scheme of stimulus delivery and signal recording set up. Air current produced by the pump and controlled by the flow meter is constantly passing through 1 of the 5 bottles, either the control bottle with paraffin oil or 1 of the 4 bottles with different concentrations of ethyl acetate in paraffin oil. A combination of the 4 valves, controlled by computer, allows one to choose among them. An electrical signal is recorded from the antenna and after amplification (AMP) stored in the computer simultaneously with the stimulus signal.

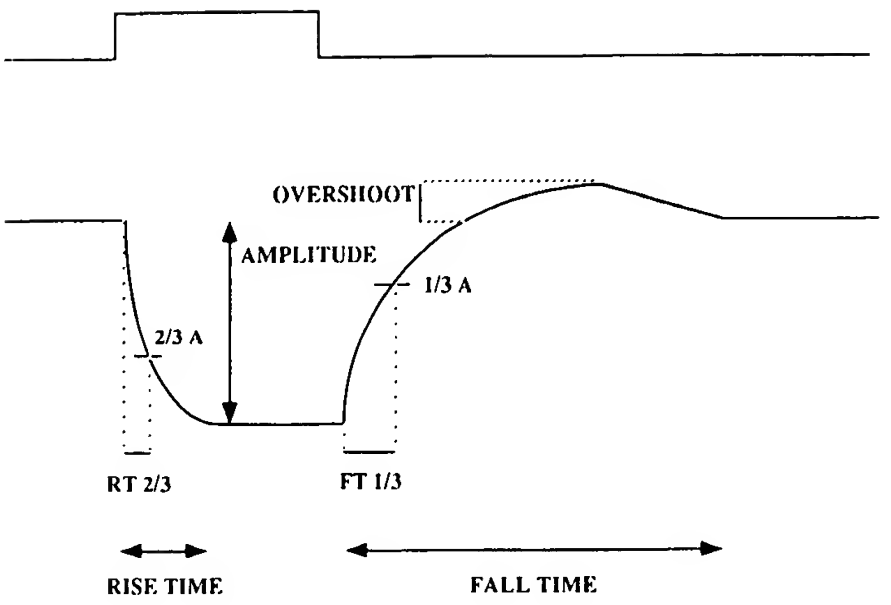


FIG. 2. Scheme of the time course of the EAG and measured parameters, amplitude, rise time constant (τ_r), fall time constant (τ_f), and relative overshoot.

occasionally observed. With this aim, the linear fit obtained from voltage data of the 2 s before stimulation and the two last seconds of each repetition (20, 30, or 60 s later, respectively, depending on duration of stimulation) was taken as baseline and subtracted from the stored data for each repetition. The new corrected data were then used to calculate amplitude and kinetic parameters (Fig. 2).

Amplitude was calculated as the maximal deflection of the potential during the stimulation time minus the average potential within the 2 s preceding the stimulus.

Rise and fall time constant values were obtained for each fly. Rise time constants were calculated as the time required to achieve two-thirds of the maximal amplitude. Fall time constants were estimated as the time necessary to recover to one-third of the maximal amplitude after stimulation (Fig. 2). In the case that 10 responses were taken for each concentration and fly, rise and fall time constants were calculated for each fly over the average of normalized EAGs. Normalization was obtained for each response by dividing voltage values by the amplitude value achieved at the end of the odorant stimulation for that response, i.e., at the time the odor pulse was turned off.

Mutant isolation and analysis

FLIES. *od A* is a chromosome II mutant obtained after hybrid dysgenic crossing of Canton-S females by males of a P-cytotype line, *Y85b1* (kindly provided by Engels, Madison WI). Another P line with a balanced second chromosome [*Cy*O (P), $S^2 cn^p bw/\pi^2$; π^2, also provided by Engels] was used for chromosome II homozygosis and stabilization of the mutation in a P-cytotype background. As a result of the crossing scheme utilized (Fig. 3), the *od A* second chromosome was derived from the Canton-S line, whereas the rest of the genomic background came from both the Canton-S as well as the P-cytotype line. Because the genetic background may affect the expression of the mutation, in addition to the Canton-S line another control line was used, a control-P line. It was obtained by crossing the two P lines used for the mutagenesis and homozygosis. This P-control line was maintained by mass crossing for ~10 generations before the test.

BEHAVIORAL TEST. Adult flies were tested in a Y-maze (Fig. 4*A*), where they were given the choice between charcoal-filtered air and different concentrations of ethyl acetate. The olfactory index (IO)

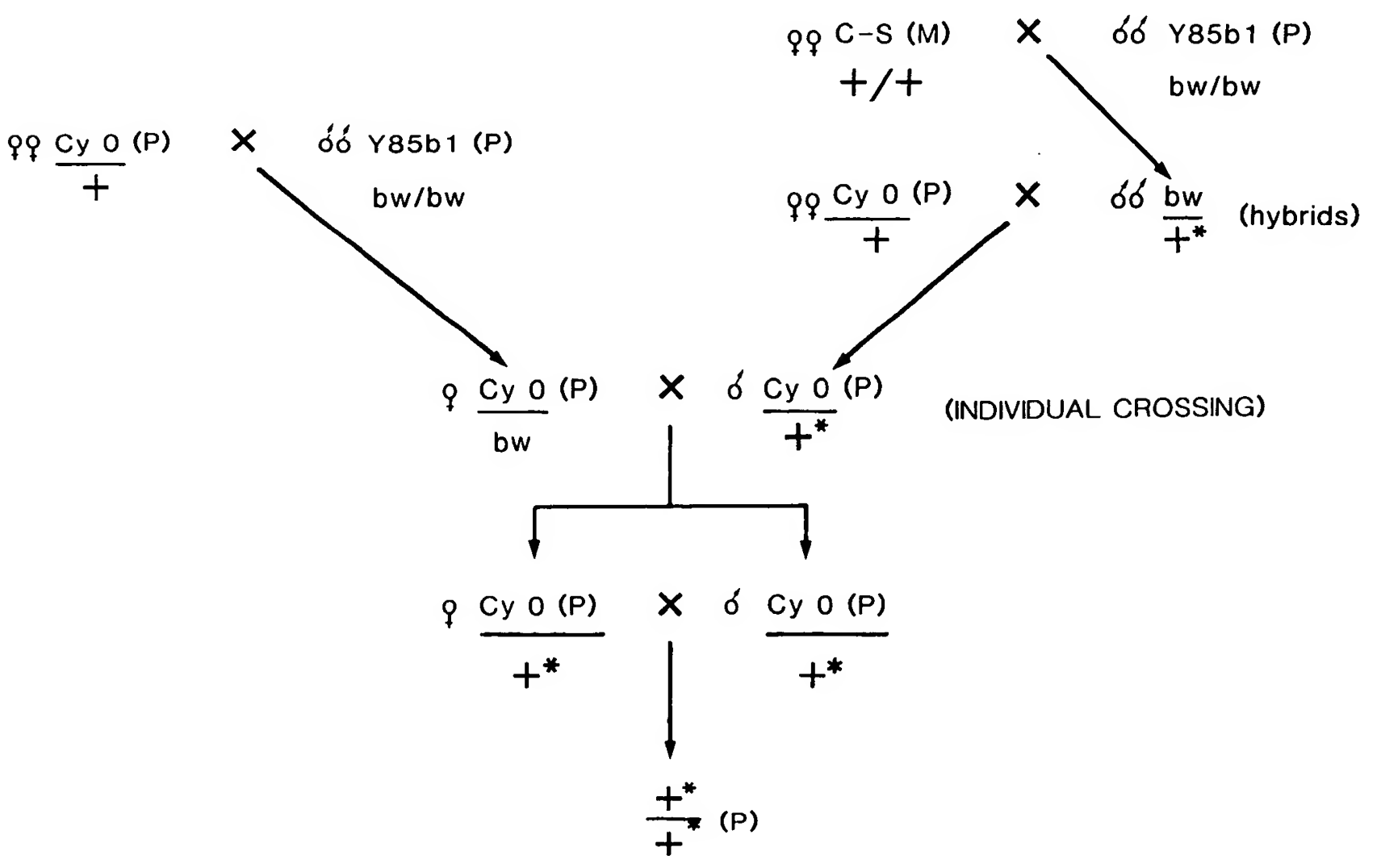
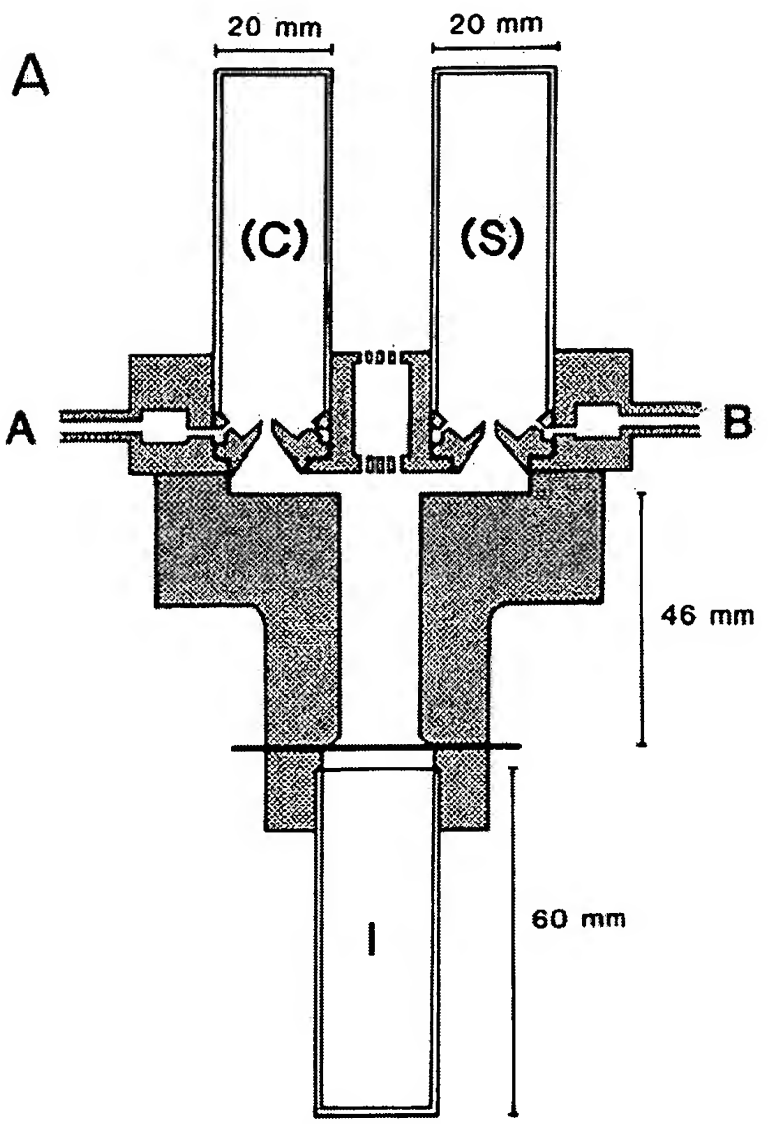


FIG. 3. Crossing scheme employed for mutagenesis and homozygosis of the chromosome II.



$$IO = \frac{S}{S + C}$$

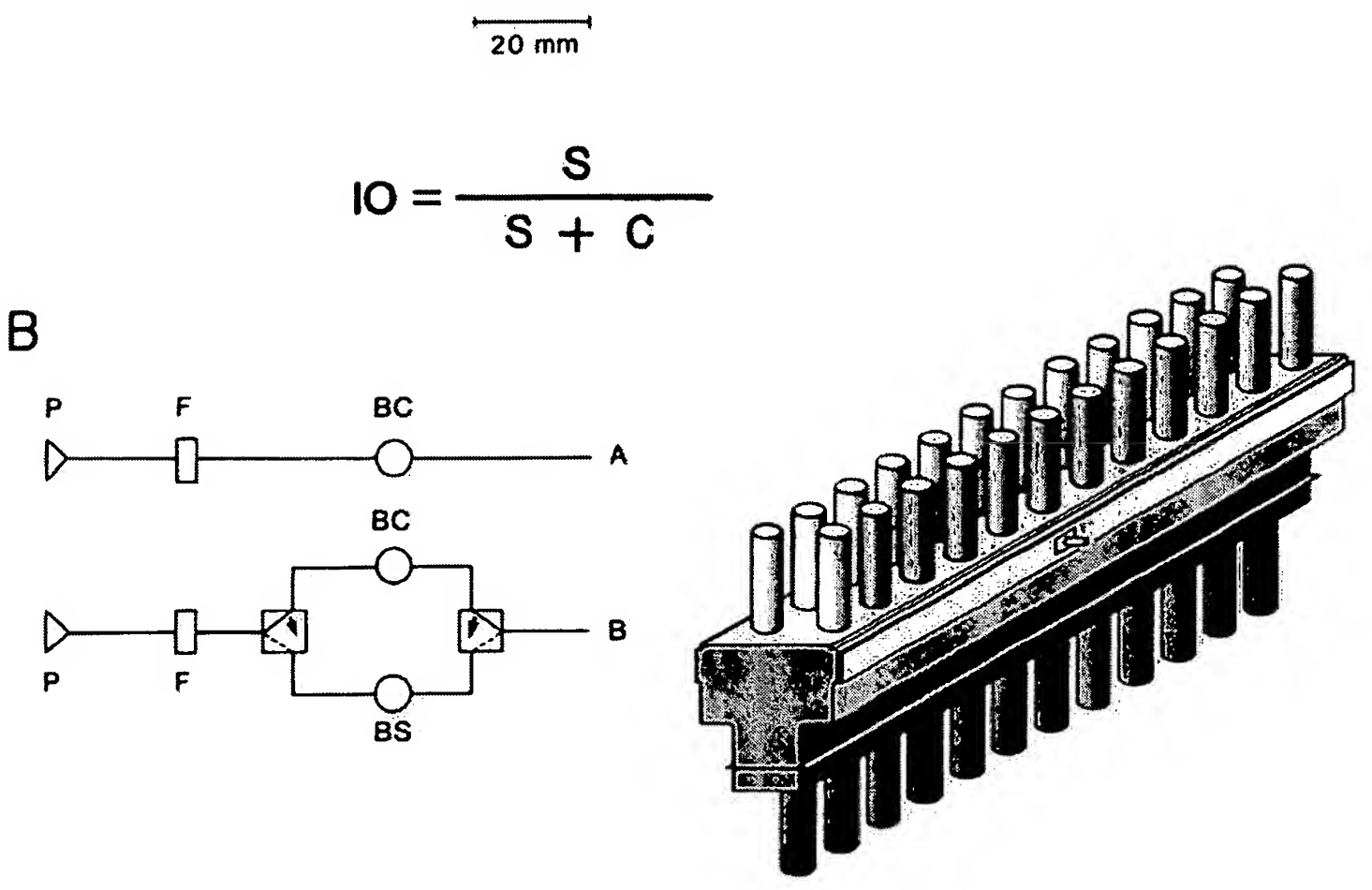


FIG. 4. *A*: Y-maze section showing a single labyrinth. I, initial tube; S, stimulus tube; C, control tube. The olfactory index (IO) describes preference between the 2 arms of the maze. IO is calculated as the number of flies that choose the stimulus tube S divided by the flies that move to either the stimulus S or the control tube C. *B, right*: Y-maze battery composed by addition in parallel of 12 single labyrinths. *Left*: stimulant and clean air current generation. P, air pump; F, flow meter; BC, control bottle with 5 ml of paraffin oil; BS, stimulant bottle with 5 ml of ethyl acetate. Air currents are connected to the sides of the Y-maze battery.

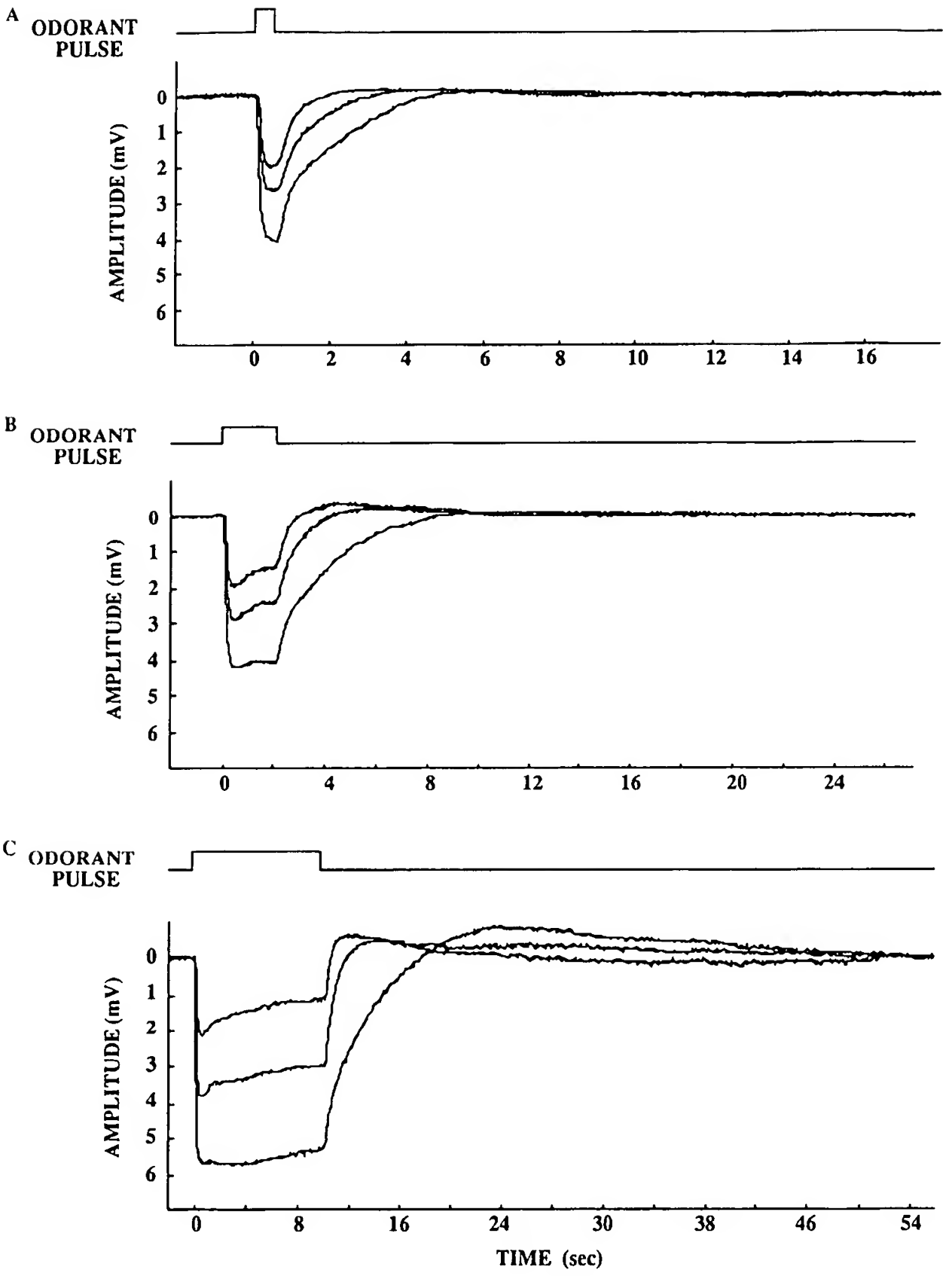


FIG. 5. Recorded signals in response to (*A*) 0.5 s, (*B*) 2 s, and (*C*) 10 s of stimulation with 3 concentrations of ethyl acetate (10^{-4}, 10^{-3}, and 10^{-2} in order from *top* to *bottom*). *Top trace* in *A–C* is stimulus. Recordings represent an average of 100 EAGs (10 repetitions per fly over a total of 10 flies) for the 0.5- and 2-s conditions (*A* and *B*) and an average of 20 EAGs (1 repetition per each of 20 flies) for the 10-s stimulation (*C*). Data have not been normalized before averaging.

TABLE 1. *Dependence of EAG amplitude on odorant concentration*

Duration of stimulation, s	Amplitude, mV† Concentration* 10^{-4}	10^{-3}	10^{-2}	Analysis of Variance Source of variation	df	SS
0.5	2.18 ± 0.21	2.96 ± 0.32	4.24 ± 0.45	Between concentrations	2	21.52‡
				Within concentrations	27	31.61
2.0	2.12 ± 0.19	3.08 ± 0.27	4.39 ± 0.44	Between concentrations	2	25.95‡
				Within concentrations	27	27.34
10.0	2.32 ± 0.12	3.97 ± 0.23	6.13 ± 0.46	Between concentrations	2	142.43‡
				Within concentrations	57	104.93

Values are means ± SE; values represent variation among flies. df, degrees of freedom; SS, sum of squares. *Concentration of ethyl acetate. †Amplitude was directly calculated from the voltage data (nonnormalized). ‡$P < 0.001$.

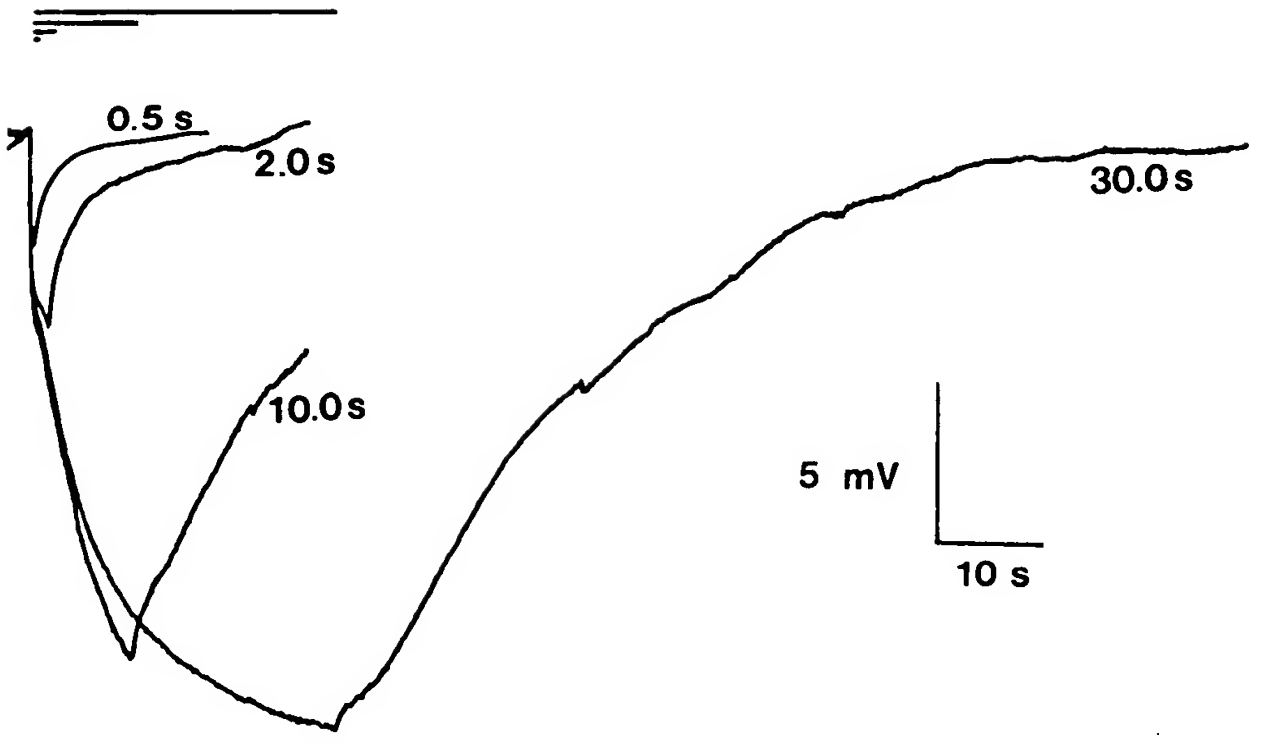


FIG. 6. Recorded signals in response to 0.5, 2, 10, and 30 s of a 10^{-1} concentration of ethyl acetate. Recordings represent an average of the voltage data obtained from 10 different flies. Upper traces show duration of stimulation.

was calculated as the ratio of the number of flies that choose the stimulus tube to the total number of flies that arrive at the end of the labyrinth. This index ranges between 0 and 1, where values between 0 and 0.5 and between 0.5 and 1 represent repellent and attractant responses, respectively. Thus the 0.5 value can be interpreted as insensitivity to the stimulus or as intermediate between attraction and repulsion.

A special Y-maze (designed by Goetz) composed of a battery of single mazes was developed to screen for abnormal olfactory preference after mutagenesis (Fig. 4*B*). The maze was used to measure a large number of lines separately but simultaneously and, after the isolation of the mutant, to characterize the whole dose-response curve. The olfactory stimulus was provided by the use of air currents and was identical for all the lines simultaneously tested. Two conduits, one through each side of the special Y-maze, connected to each individual maze arm and provided either clean or stimulant air. The air flow in each side (5 l/h) was controlled by a flow meter. Different concentrations of stimulus were achieved by mixing air coming through two wash bottles, one of them containing ethyl acetate (BS) and the other, a control bottle containing paraffin oil (BC). An electrically activated three-way air valve (LEE, LFAA 1200118H) directed the air to the intake of one or the other bottle, and concentrations depended on the ratio of the two output times. The whole system was connected 15 min before the beginning of the test to stabilize the stimulus concentration in the mazes.

Thirty individuals, starved for 24 h, were introduced into each initial tube. A separation plate prevented the flies from entering the maze at that moment. After a few minutes the plate was taken away, and the response was examined after 90 min. Sixteen repetitions for each concentration were carried out to complete the dose-response curve. Males and females were tested separately to avoid possible pheromone effects.

EAGs. Response to different ethyl acetate concentrations (10^{-4}, 10^{-3}, 10^{-2}) and three stimulation durations (0.5, 2, and 10 s) were measured in the mutant *od A* as well as in the two control lines, Canton-S and Control P. Experiments were performed in a rotatory way measuring one fly of each strain, and so on, until 10 flies for 0.5 and 2 s of stimulation and 20 flies for the 10-s condition had been tested. With this method, confusion between differences among lines and those due to variability among experimental sets can be prevented. The number of repetitions for each concentration and stimulation duration and the rest of experimental conditions were the same as previously described.

RESULTS

EAG measurements

VOLTAGE DATA. Voltage data from wild-type flies for different experimental conditions are plotted in Fig. 5. Each curve represents averaged data from 100 EAGs (10 repetitions of each odorant dose for each of 10 flies) at all concentrations for 0.5- and 2-s stimulations, and an average of 20 EAGs (1 per fly) in the 10-s stimulation condition. In cases where multiple repetitions were tested per fly and concentration, variation among repetitions was always smaller than among different flies.

TABLE 2. *Kinetic measures and relative overshoot are independent of amplitude at each stimulus condition*

Duration of Stimulation, s	Concentration								
	Rise Time Constant			Fall Time Constant			Relative Overshoot		
	10^{-4}	10^{-3}	10^{-2}	10^{-4}	10^{-3}	10^{-2}	10^{-4}	10^{-3}	10^{-2}
0.5	−0.023	−0.008	0.001	0.014	0.059	0.041	−0.016	0.013	−0.0002
2	−0.032	−0.003	0.002	−0.022	0.058	−0.109	−0.029	−0.013	−0.005
10	0.004	−0.033	−0.004	−0.026	−0.063	−0.356	0.071	0.017	0.036

Values shown are slopes of regression lines of time constants or relative overshoot over amplitude; all values are nonsignificant.

TABLE 3. *Rise time constant decreases with increasing odorant dose*

Duration of stimulation, s	Rise Time Constant, s			Analysis of Variance†		
	Concentration*					
	10^{-4}	10^{-3}	10^{-2}	Source of variation	df	SS
0.5	0.174 ± 0.008	0.156 ± 0.007	0.112 ± 0.006	Between concentrations	2	0.020‡
				Within concentrations	27	0.014

Values are means ± SE; values represent variation among flies. Abbreviations, see Table 1. *Concentration of ethyl acetate. †Analysis of variance of rise time constant values. ‡$P < 0.001$.

For all stimulation durations, peak amplitude increased with increasing concentrations of ethyl acetate (Table 1). The response achieved maximal amplitude before 0.5 s of stimulation. However, with strong stimulation some differences were found. In response to the 10^{-1} concentration, a stationary plateau was not achieved even after 30 s of stimulation (Fig. 6), and extremely large signals (in some cases >30 mV) could be recorded. In principle, the fact that the voltage level continues to polarize even after 2 s of stimulation could be interpreted as the reaction of very slow receptors or receptors specific for other odorous molecules that react to very high concentrations of ethyl acetate. However, continuation of polarization after 30 s, achieving values of >30 mV, seemed more likely to correspond to other physical changes not related to olfaction. Therefore this concentration was not used later for calculating amplitude or kinetic parameters.

An analysis of variance (Table 1, *left*) of the peak amplitude measures was carried out to check the statistical significance of the amplitude changes when concentration of odorant was increased. Because the three stimulation durations were tested in three different experimental sets and some variation in amplitude measures was previously detected among experimental sets, comparisons were limited within each set. So, although amplitude values seemed higher when 10 s of odorant were supplied, it can not be explained by increasing of response between 2 and 10 s (Fig. 5) but by variability among experimental sets. A highly significant effect ($P < 0.001$) of increasing odorant concentration over EAG amplitude values was found, in the direction of increasing amplitude. This fact agrees with the previous reports on *Drosophila* EAGs (Borst 1984; Venard and Pichon 1981, 1984).

KINETIC MEASURES. Kinetic values were shown to be very consistent throughout different sets of experiments, much more than amplitude values and independent of amplitude for each concentration of odorant. Regression lines of rise time, fall time, and overshoot parameters over the corresponding amplitude values in each fly were calculated within each concentration and duration (Table 2). In all cases, the slope of the regression line was not statistically different from zero. This means that small differences in amplitude dependent on the location of the electrode did not affect kinetic measures.

Rise time constants were calculated from the voltage data at 0.5 s of stimulation, because this time was shown to be enough to achieve the maximal amplitude except at a 10^{-1} concentration.

Table 3 shows a very significant ($P < 0.001$) decrease of rise time values to increasing concentrations of ethyl acetate.

Because a direct measurement of the number of odorant molecules that reach the fly was not taken, description of the stimulation dynamics is not available. However, for the experimental air flow used, the air volume in the intermediate tubes delivering odorant to the fly changes six times in 0.5 s. Thus changes of the stimulus concentration are not likely to explain the rise curve pattern.

Fall time constants (Table 4) were calculated for the different concentrations at each stimulation duration to study the effects of supplying more odorant molecules in different ways. Contrary to the rise time constants, fall time constants increased very significantly ($P < 0.001$, Table 4) with increasing concentrations of odorant. At the 10^{-2} concentration, fall time constant also increased in response to increasing time of stimulation. For the other two concentra-

TABLE 4. *Fall time constant increases with odorant dose*

Duration of stimulation, s	Fall Time Constant, s			Analysis of Variance†		
	Concentration*					
	10^{-4}	10^{-3}	10^{-2}	Source of variation	df	SS
0.5	0.534 ± 0.021	0.936 ± 0.042	1.618 ± 0.075	Between concentrations	2	6.01‡
				Within concentrations	27	0.70
2.0	0.480 ± 0.013	0.872 ± 0.043	2.646 ± 0.0345	Between concentrations	2	26.64‡
				Within concentrations	27	10.90
10.0	0.534 ± 0.032	1.020 ± 0.082	4.847 ± 0.533	Between concentrations	2	213.31‡
				Within concentrations	57	100.06

Values are means ± SE; values represent variation among flies. Abbreviations, see Table 1. *Concentration of ethyl acetate. †Analysis of variance of fall time constant values. ‡$P < 0.001$.

TABLE 5. *Relative overshoot depends on duration and concentration of stimulation*

Duration of stimulation, s	Relative Overshoot: Concentration* 10^{-4}	10^{-3}	10^{-2}	Analysis of Variance† Source of variation	df	SS
0.5	0.162 ± 0.021	0.105 ± 0.014	0.062 ± 0.008	Between concentrations	2	0.051‡
				Within concentrations	27	0.063
2.0	0.334 ± 0.015	0.163 ± 0.016	0.078 ± 0.009	Between concentrations	2	0.340‡
				Within concentrations	27	0.050
10.0	0.787 ± 0.146	0.290 ± 0.030	0.227 ± 0.042	Between concentrations	2	3.724‡
				Within concentrations	57	9.032

Values are means ± SE; values represent variation among flies. Abbreviations, see Table 1. *Concentration of ethyl acetate. †Analysis of variance of relative overshoot values. ‡$P < 0.001$.

tions no changes in fall time constant were perceptible; however, another parameter should also be considered. Recovery after odorant stimulation showed a certain overshoot before stabilizing to the initial potential level. Overshoot values measured as relative overshoot over normalized EAGs were highly dependent on concentration ($P < 0.001$) and time of stimulation but independent on absolute amplitude values. So, increasing concentration of odorant decreased overshoot values, but increasing duration of stimulation increased them (Table 5). If the whole recovery curve, including the overshoot, reflects some active process, increasing the number of odorant molecules hitting the antenna either by increasing concentration or time of stimulation delays the recovery to baseline.

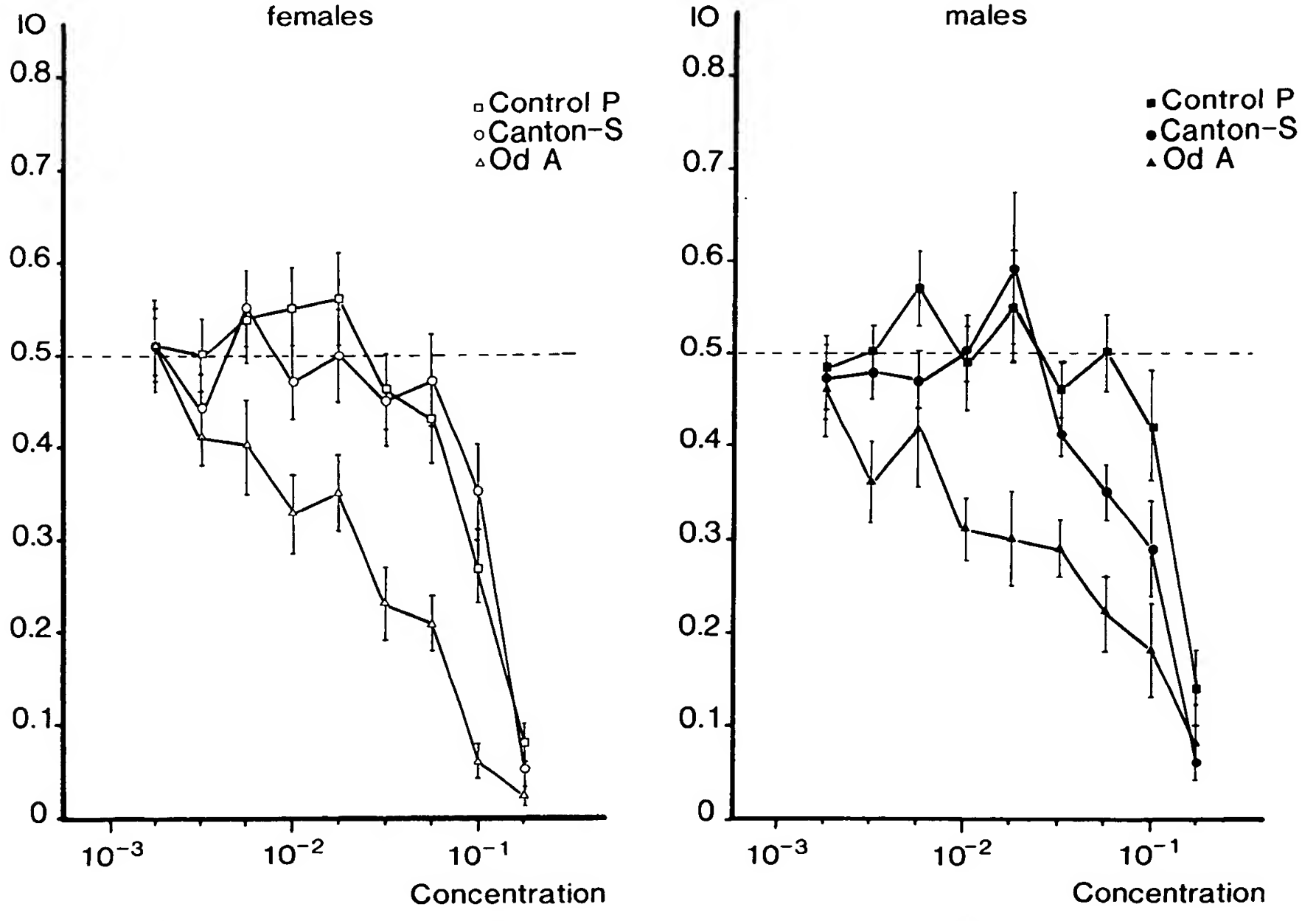
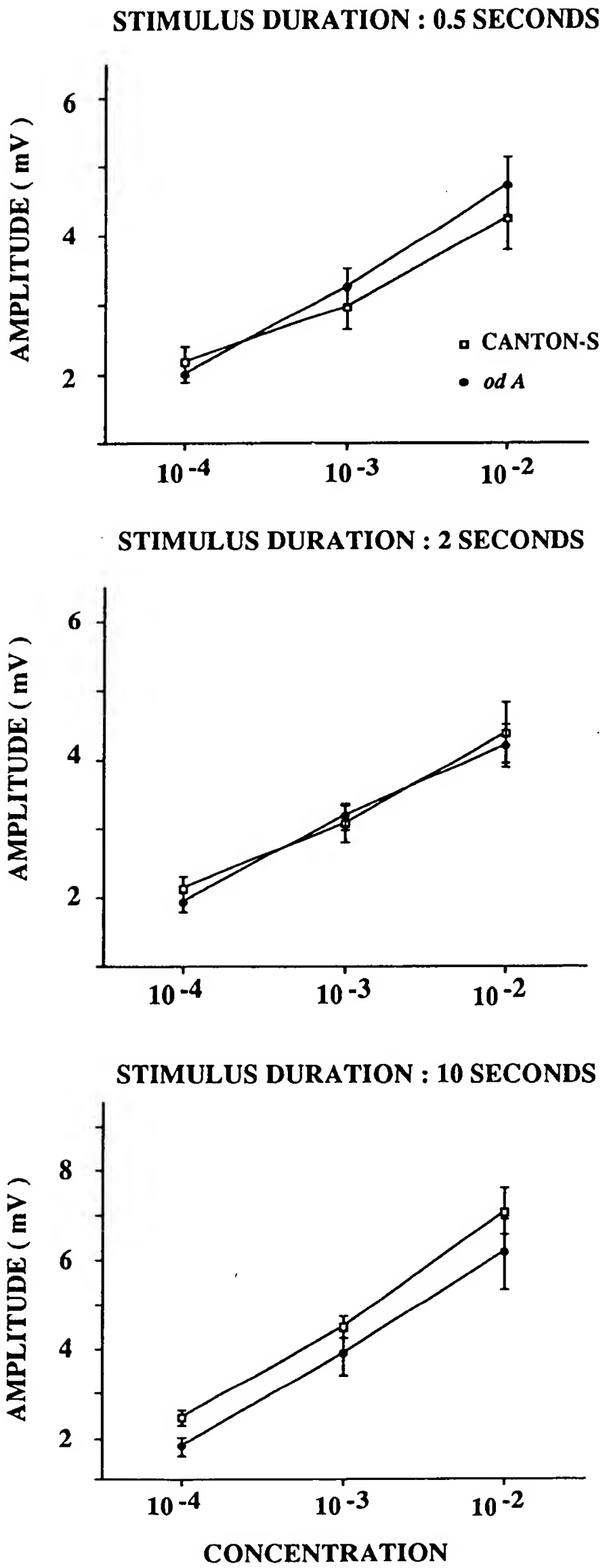


FIG. 7. Dose-response curves of olfactory response to ethyl acetate of the control lines (Canton-S and Control P) and the *od A* mutant. Average and standard error of the mean of 16 repetitions are obtained for each line and concentration.



Olfactory mutant od A

BEHAVIORAL RESPONSE. The *od A* strain showed abnormal behavior in comparison to the control lines. The complete dose-response curve displayed an enhanced repellent response with respect to the Canton-S and the Control P lines (Fig. 7). The mutant phenotype was expressed in females as well as in males.

With the current behavioral measuring techniques, an attractive effect of ethyl acetate was not found, even in the control lines, although this odorant has been described as attractive under different conditions (Rodrigues and Siddiqi 1978). But, in any case, differences between normal and mutant lines were expressed in response to every concentration in the range measured. Moreover, response in the mutant line like that of the control lines was dependent on stimulus concentration, implying that the mutant is also able to receive, transmit, and respond to concentration information.

EAG RESPONSE. EAGs obtained from the *od A* line in response to ethyl acetate have been analyzed for amplitude as well as time course of response.

The mutant line *od A* showed the same kind of response to odorant stimulation as those observed in the control lines. An increasing amplitude of voltage change was recorded from *od A* in response to increasing concentrations of ethyl acetate, and this effect was directly correlated to the time required for recovery after stimulation. The maximal amplitude was achieved in 0.5 s as for the wild-type flies.

A qualitative difference of the mutant EAGs versus those of the control lines was not expected, considering its behavioral phenotype. A quantitative difference was found in the preference of the odor, but (as in the control lines) this response was concentration dependent in *od A* (repellency increased with concentration). Therefore quantitative analysis of the EAG appears to be necessary to determine a possible peripheral effect of the mutation.

Amplitude. Amplitude values of *od A* EAGs coincided with those of the Canton-S and Control P lines (Fig. 8). An analysis of variance of the maximal amplitude data was carried out for each stimulation duration. No significant differences in amplitude were found between the mutant and the two control lines.

Kinetic measurements. In *od A*, as in the control lines, decrease of rise time (τ_r) values was observed when increasing concentrations of odorant were tested. However, at every concentration, τ_r values were greater than the corresponding values of both control lines (Table 6); the difference becomes smaller for increasing concentrations, as for the behavioral results. In other words, the voltage change in the mutant was retarded with respect to the normal phenotype. Figure 9 shows the *od A* rise time curve compared to the control Canton-S when EAGs were normalized at the end of odorant stimulation. A different shape in the EAG can also be observed.

Rise time constant values were calculated for the three

FIG. 8. Mean amplitude values and standard error of the mean in millivolts of the EAG response to (*top*) 0.5, (*middle*) 2, and (*bottom*) 10 s of 3 ethyl acetate concentrations.

TABLE 6. *Rise time constant (in seconds) of the mutant od A compared with the 2 control lines*

Duration of Stimulation, s	Line	Concentration 10^{-4}	Concentration 10^{-3}	Concentration 10^{-2}	Analysis of Variance: Source of variation	df	SS
0.5	Canton-S	0.174 ± 0.008	0.156 ± 0.007	0.112 ± 0.006	Line	2	0.017†
	Control P	0.170 ± 0.010	0.158 ± 0.006	0.106 ± 0.010	Concentration	2	0.087†
	od A	0.216 ± 0.009*	0.188 ± 0.005*	0.122 ± 0.006	Interaction	4	0.003
					Error	81	0.048
2	Canton-S	0.164 ± 0.015	0.154 ± 0.009	0.110 ± 0.010	Line	2	0.016†
	Control P	0.180 ± 0.013	0.160 ± 0.010	0.108 ± 0.007	Concentration	2	0.085†
	od A	0.208 ± 0.009*	0.194 ± 0.006*	0.120 ± 0.007	Interaction	4	0.004
					Error	81	0.082
10	Canton-S	0.200 ± 0.013	0.130 ± 0.027	0.111 ± 0.006	Line	2	0.022‡
	Control P	0.188 ± 0.010	0.163 ± 0.008	0.105 ± 0.006	Concentration	2	0.258†
	od A	0.223 ± 0.015	0.180 ± 0.007*	0.115 ± 0.008	Interaction	4	0.017
					Error	170	0.558

Values are means ± SE; values are calculated from data stored at 50 Hz for 0.5 and 2 s and 20 Hz for 10 s of stimulation (change in sample rate changes the accuracy of the measure). Abbreviations, see Table 1. *Value is significantly different ($P < 0.05$) from control values. †$P < 0.001$. ‡$P < 0.05$.

durations of stimulation, although a priori no difference among them is expected because the minimal checked time (0.5 s) suffices to achieve the maximal amplitude. Highly significant differences ($P < 0.001$) were detected among lines for 0.5 and 2 s and also ($P < 0.05$) for 10 s of stimulation. The difference in significance in both cases can be easily explained by the sampling rate, 50 and 20 Hz, respectively, which allows more accuracy for the 0.5- and 2-s measures than for the 10 second one. Moreover, rise time values for 0.5 and 2 s were calculated in each fly over a total of 10 repetitions for every concentration test, but only one repetition was done for 10 s of stimulation. This fact also affects accuracy.

Mutant electrical response showed a tendency to retarded recovery after odorant stimulation with respect to the normal flies (Table 7). Although single comparisons for each concentration failed to show statistically significant differences (except for some isolated cases), analysis of variance for each stimulation duration detected significant differences for the mutant when all the concentrations are considered ($P < 0.001$ for 0.5 s and $P < 0.05$ for 10 s). The delayed recovery tendency is displayed in Fig. 9, *A–C*, where the averages of normalized EAGs for different lines are shown for every experimental condition.

Relative overshoot of normalized EAGs was also calculated for the mutant and the control lines (Table 8). *od A* EAGs appear to be normal for this measure, and only differences in concentration or time of stimulation seemed to affect this parameter for the mutant as well as for the control lines.

All these facts support the idea that the mutation is peripherally expressed and affects kinetics but not maximal voltage. This is true for the peak amplitude as well as relative overshoot, the two voltage-related measures analyzed in the EAGs. The time-related parameters are the ones affected in the mutant.

TABLE 7. *Fall time constant (in seconds) of the mutant od A compared with the 2 control lines*

Duration of Stimulation, s	Line	Concentration 10^{-4}	Concentration 10^{-3}	Concentration 10^{-2}	Analysis of Variance: Source of variation	df	SS
0.5	Canton-S	0.534 ± 0.021	0.936 ± 0.042	1.618 ± 0.075	Line	2	1.167†
	Control P	0.598 ± 0.092	0.844 ± 0.041	1.438 ± 0.086	Concentration	2	16.376‡
	od A	0.726 ± 0.053	1.084 ± 0.055*	1.876 ± 0.199	Interaction	4	0.287
					Error	81	6.388
2	Canton-S	0.480 ± 0.013	0.872 ± 0.043	2.646 ± 0.345	Line	2	1.207
	Control P	0.568 ± 0.054	1.122 ± 0.096	3.046 ± 0.337	Concentration	2	91.879‡
	od A	0.624 ± 0.042	1.106 ± 0.087	3.004 ± 0.420	Interaction	4	0.256
					Error	81	38.928
10	Canton-S	0.534 ± 0.032	1.020 ± 0.082	4.847 ± 0.533	Line	2	30.69§
	Control P	0.338 ± 0.010	0.900 ± 0.008	4.403 ± 0.006	Concentration	2	815.92‡
	od A	0.729 ± 0.115	1.092 ± 0.067	6.835 ± 0.739	Interaction	4	31.152
					Error	170	588.547

Values are means ± SE; values are calculated from data stored at 50 Hz for 0.5 and 2 s and 20 Hz for 10 s of stimulation. Abbreviations, see Table 1. *Value is significantly different ($P < 0.05$) from control values. †$P < 0.01$. ‡$P < 0.001$. §$P < 0.05$.

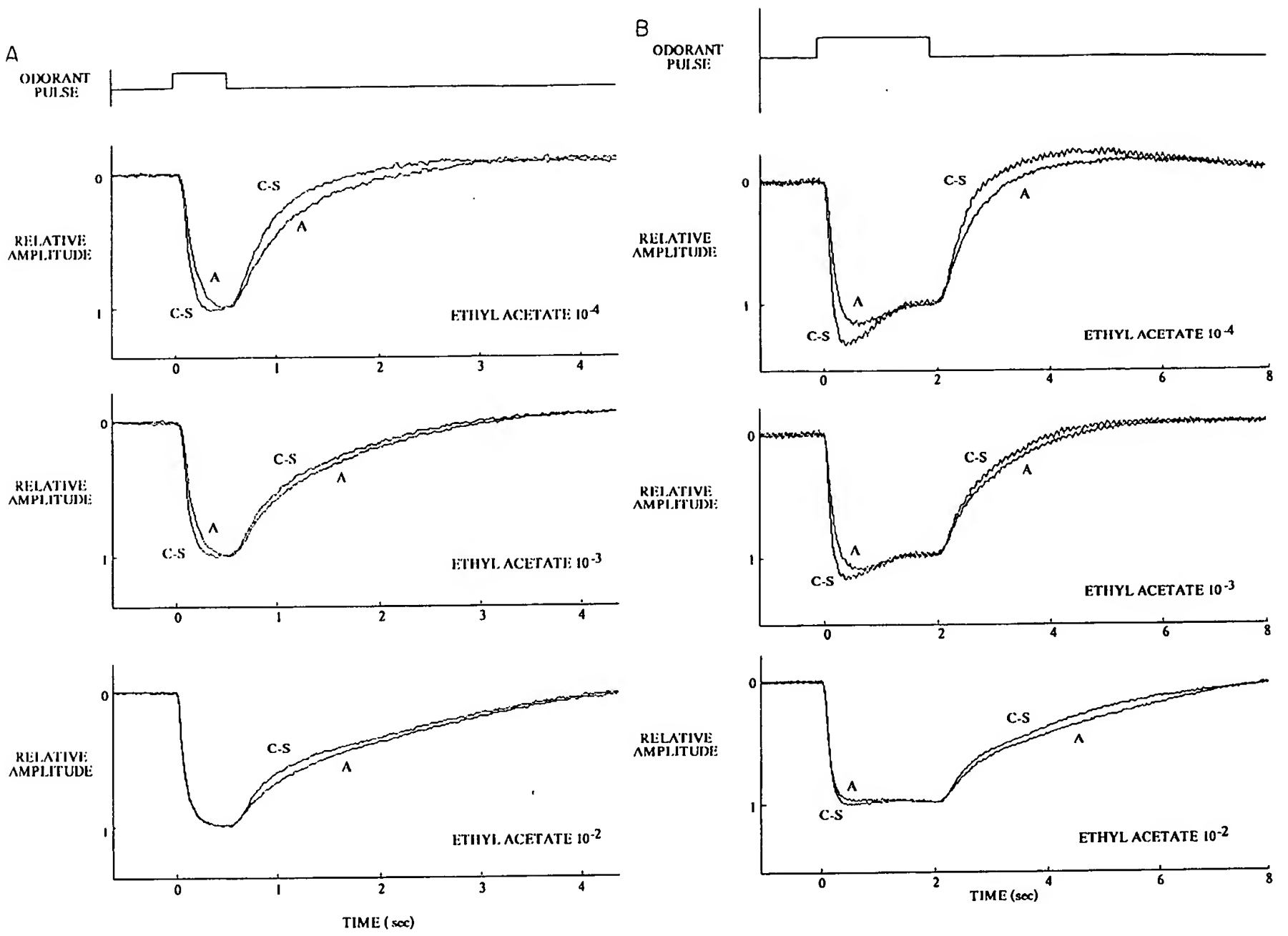


FIG. 9. EAGs obtained from *od A* and the control line Canton-S, in response to (*A*) 0.5, (*B*) 2, and (*C*) 10 s of 3 concentrations of ethyl acetate stimulation. Each trace represents the average of 100 normalized EAGs, 10 from each of 10 flies, for 0.5 and 2 s of stimulation and an average of 20 EAGs, 1 from each of 20 flies, for 10 s of stimulation.

DISCUSSION

The benefits of using the EAG for identifying mutations affecting the peripheral olfactory response have been foreseen since the first report of *Drosophila's* EAG (Venard and Pichon 1981). The possibility of correlating odorant concentration and amplitude of response also allows one to isolate mutants with a partial anosmia, assuming that amplitude in this case will be diminished compared to that of the wild-type flies. Therefore the *olf C* mutant has been described as having a peripheral effect on the basis of its diminished amplitude (Venard and Pichon 1984). However, the prospect of a mutation affecting other parameters of the EAG response and consequently the olfactory information transmitted to the CNS should also be considered.

In this report it has been shown that different concentrations of ethyl acetate produce changes in amplitude as well as in the kinetics of EAG response. Increasing concentrations of an odorous substance were correlated with an increase of amplitude of voltage change, as has been previously shown (Borst 1984; Venard and Pichon 1981, 1984), but changes in other parameters independent of amplitude can also be found. The speed of response to odorous stimulation increased with concentration while the recovery rate slowed. Recovery kinetics depended not only on the intensity but also on the duration of stimulation, suggesting dependency on the number of odorant molecules delivered. The fall time kinetics described here for *Drosophila* females in response to ethyl acetate (a general odor present in fermenting fruits) surprisingly agrees with the special case of *Bombyx mori* male's EAG in response to pheromones (Kaissling 1987). Because the EAG theoretically reflects the summed receptor potentials of many sense cells (Kaissling 1971) and not all of them are necessarily equivalent, it is difficult to correlate the measured parameters with single cellular processes happening in each olfactory neuron in response to odorants. However, the possibility of quantifying EAG responses and correlating changes in odorant stimulation with amplitude as well as with kinetic changes reinforces the idea of using the EAG as a tool for recognizing genes related to the capture and transduction processes in the olfactory sense.

Some quantitative differences have been detected in the *od A* mutant in comparison to control lines, both in behav-

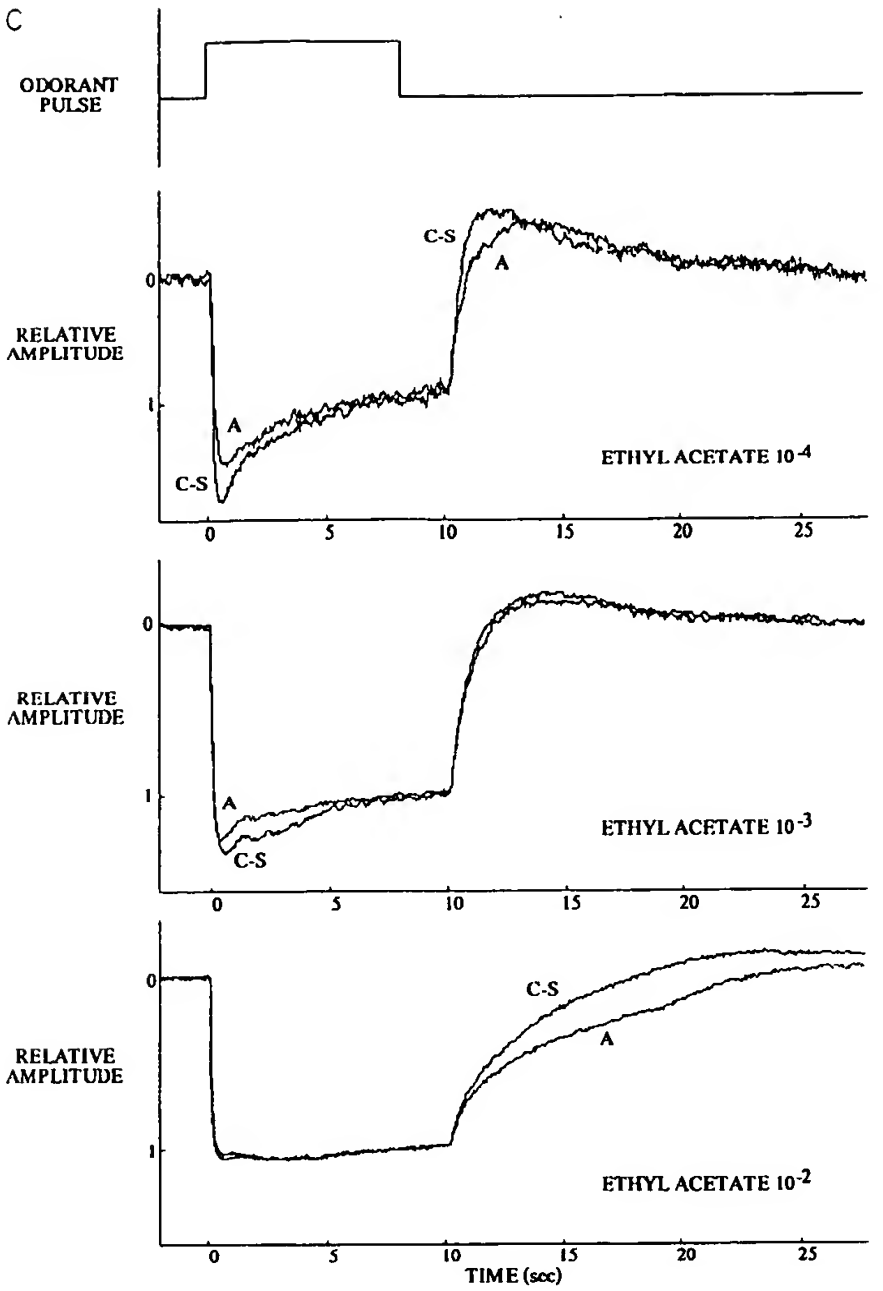


FIG. 9. *C.*

ioral response as well as in EAG phenotype. Because a complete behavioral curve with attractant and repellent responses was not found by the use of the present conditions of measurement, it is not possible to classify the effect of the mutation to one of these three categories: a general increase in sensitivity with a diminished threshold, an enhanced sensitivity but without change of threshold, or a change in response from attraction to repellency. However, one trait of the obtained phenotype should be particularly commented on. The mutant line appeared to be more sensitive than the control lines; its response to a certain concentration of stimulus corresponded to the response of the control lines to a higher concentration. This is the first example of an olfactory behavior mutant showing enhanced sensitivity.

Analysis of the EAG phenotype has revealed quantitative differences in rise time as well as in fall time constants and in the shape of the signal during stimulation. Rise time constants were very significantly delayed in the mutant response, and the differences were bigger in response to small concentrations of odorant. In this way, the rise time phenotype agrees with the described behavioral effect. The rise time probably reflects the pathway between the odorant molecule's access to the antenna and the production of changes in receptor potential. Therefore the mutation may affect some step of this process. The fall time defect, especially seen at high concentrations, could also be the result of some slower enzymatic activity. The mutant *od A* would be, then, an example of how a defect at a primary level can have a positive effect at a higher level (enhanced behavioral sensitivity). It differs from the only previously reported sensory mutation in *Drosophila* with an enhanced behavioral sensitivity, the taste mutant *gust B*, for which electrophysiological study demonstrated overproduction of receptors (Arora et al. 1987).

Correlation of behavioral and electrophysiological data is not obvious. In principle, if the behavioral defect is produced by the same gene as the electrophysiological one, changes in the EAG in the same direction as increasing concentration of odorant would be expected according to the enhanced behavioral sensitivity. Therefore, because increasing concentrations of odorant decreased rise time constant values, decrease in the mutant rise time constants would be expected. The opposite effect has been observed. On the other hand, changes in fall time response go in the expected increasing direction. Also the shape of the mu-

TABLE 8. *Relative overshoot values of the mutant od A compared with the 2 control lines*

Stimulation, s	Line	Concentration 10^{-4}	Concentration 10^{-3}	Concentration 10^{-2}	Analysis of Variance: Source of variation	df	SS
0.5	Canton-S	0.162 ± 0.021	0.105 ± 0.014	0.062 ± 0.008	Line	2	0.011
	Control P	0.164 ± 0.015	0.110 ± 0.009	0.075 ± 0.007	Concentration	2	0.158†
	od A	0.199 ± 0.025	0.126 ± 0.007*	0.083 ± 0.006	Interaction	4	0.002
					Error	81	0.16
2	Canton-S	0.334 ± 0.015	0.163 ± 0.016	0.078 ± 0.009	Line	2	0.014
	Control P	0.304 ± 0.026	0.131 ± 0.017	0.055 ± 0.006	Concentration	2	0.836†
	od A	0.293 ± 0.033	0.167 ± 0.016	0.104 ± 0.011	Interaction	4	0.015
					Error	81	0.273
10	Canton-S	0.787 ± 0.146	0.290 ± 0.030	0.227 ± 0.042	Line	2	0.067
	Control P	0.881 ± 0.179	0.236 ± 0.027	0.206 ± 0.044	Concentration	2	13.739†
	od A	0.785 ± 0.125	0.268 ± 0.037	0.138 ± 0.034	Interaction	4	0.165
					Error	170	27.337

Values are means ± SE. Abbreviations, see Table 1. *Value is significantly different ($P < 0.05$) from control values. †$P < 0.001$.

tant's EAG at small concentrations is similar to the normal EAG in response to higher concentrations.

Differences in the intensity of the defect can be found between the behavioral and electrophysiological phenotypes. On one hand, the different durations of odorant stimulation used for the behavioral and electrophysiological assays do not allow one to make direct comparisons between the intensity of the behavioral and electrophysiological defects. However, several examples in the literature have shown that quantitative changes at a cellular level can have a dramatic behavioral effect. For instance, quantitative reduction of sodium currents in the *para* mutant (Nelson and Wyman 1990; O'Dowd et al. 1989) is translated into total paralysis of the fly at increased temperatures. On the other hand, because the EAG is a summation of single neuronal effects, changes in the EAG could correspond to changes in some subgroup of antennal neurons, and this fact could make direct behavioral-electrophysiological correlation difficult. Likewise, the expression of the mutation in the antennal neurons does not preclude other possible neuronal effects in the rest of the olfactory stimuli-behavioral response pathway. Direct measures in the antennal nerve, as well as the possible identification of the gene product by the use of molecular biology techniques, appear as the next step for answering these questions and trying to understand how concentration information is transmitted and interpreted by the fly.

I thank A. Borst and K. G. Goetz for their help and suggestions during the course of the experiments. K. G. Goetz designed the battery of Y-mazes used for testing olfactory behavior. Thanks to A. Borst, R. Ayer, and J. Carlson for critically reading different versions of the manuscript.

This work was supported by a fellowship of the Spanish Ministry of Education and Science.

Address for reprint requests: E. Alcorta, Dept. of Biology, KBT 1142, Yale University, PO Box 6666, New Haven, CT 06511.

Received 24 July 1990; accepted in final form 6 November 1990.

REFERENCES

ACEVES-PIÑA, E. O. AND QUINN, W. G. Learning in normal and mutant *Drosophila* larvae. *Science Wash. DC* 206: 93–95, 1979.

ARORA, K., RODRIGUES, V., JOSHI, S., SHANBHANG, S., AND SIDDIQI, O. A gene affecting the specificity of the chemosensory neurons of *Drosophila*. *Nature Lond.* 330: 62–63, 1987.

BORST, A. Identification of different chemoreceptors by electroantennogram-recording. *J. Insect Physiol.* 30: 507–510, 1984.

GANETZKY, B. AND WU, C. F. Neurogenetics of membrane excitability in *Drosophila*. *Annu. Rev. Genet.* 20: 13–44, 1986.

HALL, J. C. Genetics of the nervous system in *Drosophila*. *Q. Rev. Biophys.* 15: 223–479, 1982.

HALL, J. C. AND GREENSPAN, R. J. Genetic analysis of *Drosophila* neurobiology. *Annu. Rev. Genet.* 13: 127–195, 1979.

HEISENBERG, M. Genetic approach to a visual system. In: *Handbook of Sensory Physiology*, edited by H. Autrum. Berlin: Springer-Verlag, 1979, p. 665–679.

HEISENBERG, M. AND GOETZ, K. G. The use of mutations for the partial degradation of vision in *Drosophila melanogaster*. *J. Comp. Physiol.* 98: 217–241, 1975.

HELFAND, S. L. AND CARLSON, J. R. Isolation and characterization of an olfactory mutant in *Drosophila* with a chemically specific defect. *Proc. Natl. Acad. Sci. USA* 86: 2908–2912, 1989.

KAISSLING, K. E. Insect olfaction. In: *Handbook of Sensory Physiology. Chemical Senses. I. Olfaction*, edited by L. M. Beidler. Berlin: Springer-Verlag, 1971, p. 351–431.

KAISSLING, K. E. *Wright Lectures on Insect Olfaction*, edited by K. Colbow. Burnaby, B. C., Canada: Simon Fraser Univ., 1987.

MCKENNA, M., MONTE, P., HELFAND, S. L., WOODARD, C., AND CARLSON, J. R. A simple chemosensory response in *Drosophila* and the isolation of *acj* mutants in which it is affected. *Proc. Natl. Acad. Sci. USA* 86: 8118–8122, 1989.

NELSON, J. C. AND WYMAN, R. J. Examination of paralysis in *Drosophila* temperature-sensitive paralytic mutations affecting sodium channels: a a proposed mechanism of paralysis. *J. Neurobiol.* 21: 453–469, 1990.

O'DOWD, D. K., GERMERAAD, S. E., AND ALDRICH, R. W. Alterations in the expression and gating of *Drosophila* sodium channels by mutations in the *para* gene. *Neuron* 2: 1301–1311, 1989.

PAK, W. L. Mutants affecting the vision in *Drosophila melanogaster*. In: *Handbook of Genetics*, edited by R. C. King. New York: Plenum, 1975, p. 703–733.

PAK, W. L. Study of photoreceptor function using *Drosophila* mutants. In: *Neurogenetics: Genetic Approaches to the Nervous System*, edited by X. O. Breakfield. New York: Elsevier/North-Holland, 1979, p. 67–99.

POWER, M. E. The antennal centers and their connections with the brain of *Drosophila melanogaster*. *J. Comp. Neurol.* 85: 485–509, 1946.

RODRIGUES, V. AND SIDDIQI, O. Genetic analysis of chemosensory pathway. *Proc. Indian Acad. Sci. Sect. B* 87: 147–160, 1978.

SIDDIQI, O. Neurogenetics of olfaction in *Drosophila melanogaster*. *Trends Genet.* 3: 137–142, 1987.

VENARD, R. AND PICHON, Y. Etude electro-antennographique de la response peripherique de l'antenne de *Drosophila melanogaster* a des stimulations odorantes. *C. R. Acad. Sci. Paris* 293: 839–842, 1981.

VENARD, R. AND PICHON, Y. Electrophysiological analysis of the peripheral response to odours in wild type and smell-deficient *olf C* mutant of *Drosophila melanogaster*. *J. Insect Physiol.* 30: 1–5, 1984.

WOODARD, C., HUANG, T., SUN, H., HELFAND, S. L., AND CARLSON, J. Genetic analysis of olfactory behavior in *Drosophila*: a new screen yields the *ota* mutants. *Genetics* 123: 315–326, 1989.